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ENGLISH SUMMARY

During the past decades molecular imprinting technique has emerged as highly putative tool and effective method in developing sensors in material science. It is a promising method to introduce synthetic receptor–like structures in crosslinking polymers. These are known as MIPs that are relatively cheap and simple, and can be prepared in tailor-made fashion. The imprinting and recognition can be performed covalently, non-covalently, or semi-covalently. In this thesis, we have focused on: molecular imprinting and recognition with the covalent disulfide bonds; optimization on the degree of crosslinking in wide range of forms of materials including organic polymers, inorganic polymers, and organic/inorganic hybrid materials ; post modification on imprinted polymers without loss of size, shape, and molecular memory of imprinted polymers; and recognition carried out in organic and aqueous solutions with fast, efficient, and high selective binding affinities.

In Chapter-2, we focused on covalently imprinted novel synthetic benzyl mercaptan receptors with tunable binding sites in organic crosslinking polymers. The functional monomer 3-benzyldisulfanylprop-1-ene (BDP) was designed, synthesized, and characterized. A series of imprinted and non-imprinted polymers were prepared by using different ratios of functional monomer/crosslinker under ultraviolet radiation in comparison with thermal cross- linking. Subsequent disulfide reduction resulted in free thiol containing binding sites throughout the polymers. Furthermore, a novel post modification method has been employed to alter the binding sites which facilitate recognition on the templates by forming reversible disulfide bonds through thiol-disulfide exchange strategy. Rebinding and selective studies on benzyl mercaptan related templates proved that rapid covalent rebinding was significantly more efficient than other range of non-covalent interactions.

In Chapter-3, a novel synthetic amino acid (modified cysteine) was prepared through covalent disulfide bond with vinyl functionality as the functional monomer, and recognition was carried out in aqueous media. This functional monomer (9-fluorenyl methoxy carbonyl)-S-(prop-2-ene-1-thiol)-L-Cysteine, (Fmoc-Cys(SCH_2CHCH_2)-OH) was prepared and co-polymerized with crosslinkers under ultraviolet light for 15 h at room temperature and additional thermal reaction for 3 h at 80 ^oC. Reduction of disulfides yielded thiol tailoring binding sites. Rebinding and selective studies in aqueous and protic solvents showed these imprinted polymers selectively recognizing templates with high binding constants via thiol-disulfide exchange reactions. Further repeated rebinding and selective studies were conducted to estimate the non-covalent and covalent recognition on templates. From these studies, we prove that polymer particles are reproducible with constant binding strengths and recognition properties.

In Chapter-4, molecular imprinted polymers of modified cysteine [(9-fluorenylmethoxycarbonyl)-S-(prop-2-ene-1-thiol)-L-Cysteine] was prepared as

organic/inorganic hybrid materials via sol-gel process followed by radical polymerization. Along with imprinted hybrids, other non-imprinted hybrid materials were synthesized and optimized for crosslinking density and incorporation of the organic network in the inorganic network. Imprinted mesoporous hybrid polymers covalently and selectively recognized the templates in water with high binding affinity. The covalent and non-covalent binding kinetics suggested that templates were covalently bonded to imprinted polymers in an efficiently short period of time. These attractive high covalent binding recognitions of sulfur containing cysteine (thiol-disulfide exchange) in hybrid materials with high selectivity in water could be used to develop models to recognize proteins in biological samples which contain cysteine residues in their structure.

In Chapter-5, imprinted nano-structured inorganic silica materials were expected more attractive over organic ones due to their good mechanical, thermal, and optical properties and their negligible swelling in mobile phases. Synthesized functional monomer 3-benzyldisulfanylpropyltrimethoxysilane imprinted silica network was successfully prepared via sol-gel process. The optimal conditions and ratios between functional monomer and crosslinker were investigated and wide range of ratios have been employed in series of syntheses. Post modified MIPs have been successfully employed without altering the binding sites' shape, size, and memory in the imprinted silica polymers. The batch rebinding and selectivity studies demonstrate the imprinting effect and higher binding affinities with reversible formation of covalent disulfide bonds.

DANSK RESUME

Molecular imprinting har udviklet sig til et anerkendt redskab og en effektiv metode til at udvikle sensorer indenfor materialevidenskab indenfor det seneste årti. Det er en lovende metode til at fremstille syntetiske receptorlignende strukturer i crosslinkede polymerer. Disse er kendt under betegnelsen MIP, som er billige, simple og kan udvikles til specielle behov. Imprinting og efterfølgende genkendelse kan enten være kovalent, ikke-kovalent eller semi-kovalent. Denne afhandling fokuserer på: molecular imprinting og genkendelse med kovalente disulfid bindinger; optimering af graden af crosslinking i en bred vifte af materialer inklusiv organiske polymerer, uorganiske polymerer og organisk/uorganisk hybrid materialer; Eftermodificering på imprintede polymerer uden tab af størrelse, form og molekylær hukommelse af de imprintede polymerer; og genkendelse i både organiske og vandige opløsninger med hurtige, effektive og meget selektive bindingsaffiniteter.

Kapitel-2 fokuserer på nye kovalent imprintede syntetiske benzyl mercaptan receptorer med justerbare bindingssites i organiske crosslinkede polymerer. 3-benzyldisulfanylprop-1-ene (BDP), en funktionel monomer, blev designet, syntetiseret og karakteriseret. En række imprintede og ikke-imprintede polymerer blev fremstillet med varierende funktionel monomer/crosslinker ratio under ultraviolet stråling, til forskel fra thermisk crosslinking. Herefter blev disulfid reduceret, resulterende i frie thiol-indeholdende bindingssites i polymerene. Derudover blev der brugt en ny eftermodificering til at ændre de bindingssites som bruges ved genkendelsen af skabelonen ved at lave reversible disulfid bindinger gennem en thiol-disulfid udveksling. Genbinding og selektivitets-studier omkring benzyl mercaptan skabeloner viste at hurtig kovalent genbinding var signifikant mere effektivt end andre ikke-kovalente interaktioner.

I kapitel-3 genemgåes en ny syntetisk aminosyre (modificeret cystein), fremstillet gennem kovalente disulfid bindinger med vinyl funktionalitet som den funktionelle polymer, hvor genkendelse blev udført i vandigt medie. Denne funktionelle monomer (9-fluorenyl methoxy carbonyl)-S-(prop-2-ene-1-thiol)-L-Cystein, (Fmoc-Cys(SCH2CHCH2)-OH) blev fremstillet og copolymeriseret med crosslinkere under ultraviolet lys i 15 timer ved stuetemperatur, og efterfølgende opvarmet til 80 oC i 3 timer. Reduktion af disulfid gav specifikke thiol bindingssites. Genbinding og selektivitets-studier i vandige og protiske opløsningsmidler viste at disse imprintede polymerer udviste selektiv genkending med høje bindingskonstanter via thiol-disulfid udvekslingsreaktioner. Yderligere genbinding og selektivitets-studier blev udført for at estimere den ikkekovalente og kovalente genkendelse af skabelonerne. Disse resultater viser at polymerpartiklerne kan genskabes med konstant bindingsstyrke og genkendelsesegenskaber.

Kapitel-4 omhandler molecular imprintede polymerer af modificeret cystein [(9-fluorenyl methoxy carbonyl)-S-(prop-2-ene-1-thiol)-L-Cystein] fremstillet som organisk/uorganisk hybrid materialer via sol-gel med efterfølgende radikal polymerisering. Udover de imprimentede hybrider blev der også fremstillet andre ikke-imprimentede hybrid materialer, som blev optimeret i forhold til cross-link densitet og inkorporering af det organiske netværk i det uorganiske netværk. Imprimentede mesoporøse hybrid-polymerer kunne genkende skabeloner kovalent og selektivt i vand med høje bindingsaffiniteter. Kinetikken for de kovalente og ikke-kovalente bindinger tydede på at skabelonerne var kovalent bundne til de imprimentede polymerer i et kort, effektivt tidsrum. Disse fordelagtigt høje kovalente bindingsgenkendelser af svovl-indeholdende cystein (thiol-disulfid ombytning) i hybrid-materialer med høj selektivitet i vand kan bane vejen for modeller til genkendelse af proteiner i biologiske prøver indeholdende cystein i deres struktur.

Kapitel-5 fokuserer på imprimentede nano-strukturerede uorganiske silica materialer, da de gode mekaniske, termiske og optiske egenskaber samt ubetydelig ekspandering i mobile faser giver visse fordele over organiske materialer. Et funktionelt monomer silica netværk bestående af 3-benzyldisulfanylpropyltrimethoxysilane blev fremstillet ved sol-gel metoden. De optimale betingelser og ratioer mellem funktionel monomer og crosslinker blev undersøgt, og brugt i en række synteser. Eftermodificering af MIP blev udført uden ændring af størrelsen, formen eller hukommelsen på bindingsstederne i de imprimentede silica polymerer. Genbinding og selektivitets-studier viser en imprinting effekt, samt højere bindingsaffinitet med reversibel dannelse af kovalente disulfidbindinger.

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Harsha Vardhan Reddy Burri

LIST OF PUBLICATIONS:

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Combining Aptamers and *In Silico* Interaction Studies to Decipher the Function of Hypothetical Proteins. Prashanth, Suravajhala; Burri, Harsha Vardhan Reddy; Heiskanen, Arto (2014). European Chemical Bulletin, 3, 809-810.

Polymerase-directed Synthesis of C5-ethynyl Locked Nucleic Acids. Veedu, Rakesh N; Burri, Harsha Vardhan; Kumar, Pawan; Sharma, Pawan K; Hrdlicka, Patrick J; Vester, Brite; Wengel, Jesper (2010). Bioorganic & Medicinal Chemistry Letters, 20, 6565-6568.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

1.1 MOTIVATION

We live in a world, with systems based on interacting each other. These interaction plays a major role in forming complex structures with atoms, molecules, macro/bio – molecules, and whole cells. Weak but sufficient non-covalent interactions keep the complex structures in their dynamic nature in association and dissociation. This kind of dynamic property is fundamental to functions of molecular recognition in biological and chemical processes. Such sophisticated systems are essential for existence of life. In general, these complex structures in biological systems interact each other via weak non-covalent interactions such as hydrogen bonding, ion pairing, van der Waal forces and hydrophobic interactions. These interactions are weaker than covalent bond, those are not existing in such complexes since strong interactions keep the atoms or molecules to the distinct targets (Komiyama et al, 2003; Yan and Ramstrom, 2005).

Nowadays wide variety of synthetic techniques are being developed to obtain molecular recognition by the mimicking the ones from nature. Among these, molecular imprinting is a promising and accepted tool to prepare template specific cavities with tailor made fashion in polymer structures. Molecular imprinting technique is a multidisciplinary field with high activity in several areas of science and technology: analytical chemistry, supramolecular chemistry, synthetic organic, inorganic, and bio-chemistry. Based on type of interactions used in the process, imprinting can be done in three major means: covalent, non-covalent, or semi-covalent imprinting approaches. Since these methods have their own advantages and limitations (Komiyama et al, 2003; Yan and Ramstrom, 2005; Sellergren et al., 2006).

For non-covalent imprinting, non-covalent interactions are attractive in self-assembly process in complex formation, rather easy in template removal process and reversible forming non-covalent interactions are attractive in recognition process. However, in this method heterogeneous binding sites are formed in polymers, shows low binding affinities, and choice of solvents for rebinding are limited (Cram and Cram., 1974; Hioki and Still., 1998; Lin et al., 2006; Kirk et al., 2009; Kempe, 2000; Matsui et al., 2009). For covalent imprinting, covalent interactions are attractive ones over non-covalent in preparing pre-polymerization adducts, and choices of solvents are wide open. However, due to the strong interactions (covalent bonding between the host and guest), removal of template is rather hard and choices of reversible bond under certain easy conditions are much limited (Wulff, Heide, and Helfmeier 1986; White et al. 2001; Kamplain and Bielawski 2006; Wulff et al., 1991; Wulff et al., 1984). Semi-covalent imprinting: combination of non-covalent and covalent interactions is used in imprinting process and recognition process. With this hybrid approach, some of the drawbacks can be overcome related to both approaches. (Takeuchi et al. 2006; Takeda

et al. 2009; Sellergren and Andersson 1990; Whitecomb et al., 1995; Kirsch et al., 2000).

As the main research focuses, three main objectives on this thesis are: i) Develope the imprinting and rebinding with covalent interactions. Wide range of covalent interactions has been examined and disulfide bonds are found attractive. Since disulfide bonds are covalent and be reversible for forming and cleavage with thiol-disulfide exchange strategy. Disulfide bond energies are comparatively higher than non-covalent bond energies and meanwhile lower than other range of covalent interactions, thus, moderately ease in both template removal and rebinding process. Based on these characteristics, covalent imprinting has been performed in benzyl mercaptan and modified amino acid; ii) Optimization on the degree of crosslinking, which plays a key role in imprinted polymer stiffness and maintain binding cavity shape. Hence, wide range amounts of crosslinkers have been used for optimizing imprinting process; iii) Materials in imprinting process: imprinting has been performed in organic polymers, inorganic polymers, and organic/inorganic hybrids. Imprinting in organic polymers is attractive since target molecules are in angstrom size, and recognitions were carried out in organic solvents. However, when target templates are in nanometer scale; best recognition can be achieved in inorganic media or in aqueous media. Imprinting in inorganic polymers is comparatively attractive over organic ones since their possible recognition in inorganic media or in aqueous media. But these inorganic polymers are rigid network and solute distribution is limited. To overcome some of these problems, combination of organic and inorganic features in hybrid materials are highly attractive; iv) Post modification on imprinted polymers is developed without loss of size, shape, and molecular memory of imprinted polymers; and v) Recognition was carried out in organic and aqueous solutions with fast, efficient, and high selective binding affinities.

1.2 NATURAL RECEPTORS

All living organisms continuously expose to nature and interact with various substances. However body gives proper response to some molecules or substances by selectively recognizing them with receptors. All organisms contain wide variety of receptors which regulate the cellular metabolism in the body. A receptor is a molecule or group of molecules that specifically receives the chemical signals from the surrounding cells or external environment and mediates the cellular responses in the organism. Naturally occurring receptors are typically found in cell surfaces and embedded with either the plasma membrane or cytoplasm. These receptors are highly selective towards their respective compounds known as ligands which include small organic compounds, hormones, proteins, microorganisms, neurotransmitters, lipid analogues, and amino acid derivatives etc.. Receptors significantly recognize the ligands and forms receptor-ligand complexes that are based on lock-and-key model. During recognition process many of non-covalent interactions takes place such as hydrogen bonding, van der Waal forces, electrostatic interactions, and hydrophobic

interactions etc.. These complexes are pronounced sufficiently stable, and irreversible, comprising substantial equilibrium constants (Hoshini et al., 2010; Bongrand, 1999; Janiak and Kofinas, 2007; Hoshini et al., 2008).

In general, receptors are made up of proteins or group of proteins and their nature and function is dictated by amino acid sequence in the proteins. Upon ligand binds to the receptor, conformational changes occur in three dimensional structure of receptor and corresponding signals pass in to cells. Many of the receptors typically interact with other proteins or other receptors and are naturally embedded with plasma membrane (Ex: 7TM transmembrane receptors). Using these receptors in analytical reagents will be very expensive and face series of complex process in interactions, and meanwhile raises issues like stability and functionality of natural ones in *in vitro* experiments. Thus, challenges for the scientists is to develop inexpensive, stable synthetic receptors that can be able to recognize the compounds such as biological, physical and chemical elements in diverse conditions like thermal, chemical, and physical states (Hoshini et al., 2008; Hoshini et al., 2010).

1.3 ARTIFICIAL RECEPTORS

The man-made mimics of synthetic receptors have greater advantages over natural receptors. The discovery of synthetic man-made receptors are opened new era in designing synthetic compounds which would form reversible, non-covalently bonded highly selective complexes. The complexes play major role in biology and in most of the complexes many of non-covalent interactions are involved in recognition. In order to design molecular recognition in synthetic receptors, proper evaluation should to be done in bonding interactions and host and guest properties. The choice of molecular design is open and wide variety of functional groups can be introduced according to our choices, which are not found in natural receptor. There exits challenges in making of synthetic ones, for example, the materials should be flexible and stable in thermal, chemical, and physical conditions and are able to recognize the elements. The ideal synthetic receptors should be inexpensive, non-toxic, easily bioavailable and biodegradable as well as containing highly sophisticated recognition sites, preferentially double or multi recognition sites (Dickert et al., 2004; Tabushi et al., 1984; Sutherland., 1990; Cram and Cram., 1974; Lehn., 1988; Sakurai et al., 1998). Some examples of man-made synthetic receptors structures are crown ethers, cyclodextrins, and molecularly imprinted polymers.

1.3.1 CROWN ETHERS

Crown ethers are the cyclic polyethers composed of the repeating ethyleneoxy units, these are widely used and firstly discovered synthetic receptors. The cyclic host accompanies the guest molecules like a crown, therefore it has been named as crown ethers. Usually, crown ethers named as: X-Crown-Y, the number of X specifies the total number of atoms in the ring and number of Y indicates the total number of

oxygen atoms in the ring. The typical crown ether molecule contains hydrogen, carbon, and oxygen atoms. Each oxygen atom binds with two carbon atoms and arranged in a ring form. The highly electro negative oxygen atom serve as a binding site for ions via dipole-ion interactions. Crown ethers are flexible molecules and obtain large number of conformations, and they are classified in to structural types: noncyclic hosts (podands), monocyclic hosts (coronands), and oligocyclic hosts (cryptands). As further developments in cryptands structure, better recognition of guest cation have been obtained (Liu et al., 2008; Mazik et al., 2006; Reichwein et al., 1994; Kuo et al., 2007).

Crown ethers are used in various fields in chemistry and biology. The complexing properties of crown ethers are their attracting uses as ion selective electrodes in sensing devices, e.g., in extraction analysis studies of metal ions (Mohapatra et al., 2009 and Kakhki and Rounaghi, 2011). The analogues of crown ethers are macrocyclic polyamines that can be obtained by replacing oxygen atoms with nitrogen atoms. These are specifically utilized in recognition of azide ions and can be immobilized onto the surfaces of electrodes for preparing ion sensing devices. The other type of macrocyclic ethers which contains sulfur atoms have been developed and employed in recognizing soft ions. Chiral nature of substituted crown ethers is exploited in separation of optically active compounds from the racemic mixtures (Salvatore et al., 2002). Since crown ethers contain high coating efficiency and unique selectivity in separation of polar compounds, they have been usually taken advantages of stationary phase in capillary gas chromatography (Jin and Fu, 1989). Although crown ethers having many attractive applications in variety of fields, they possess some limitations such as relatively expensive preparation and high synthetic workload of crown ethers, and specific recognition on the ion guests (Eastburn and Tao., 1994).

1.3.2 CYCLODEXTRINS

Cyclodextrins are naturally occurring macrocyclic host molecules which usually bind specifically with guest compounds. Various synthetic routes and methodologies have been developed to alter the desired modifications in physical and chemical properties of cyclodextrins. Significant growth has been obtained in preparing artificial cyclodextrins receptors based on chemical modifications of cyclodextrins. Especially these modifications and substitutions of desired functional groups on cyclodextrins became one of the most attractive compounds in host-guest chemistry. Cyclodextrins can be obtained from naturally occurring polysaccharide starch through certain enzymatic cleavages of polysaccharides in to cyclic oligomers. These cyclic oligomers composed of 6, 7, or 8 glucosidic units namely α-, β-, and γ-cyclodextrins. Figure 1 illustrates the chemical structure of β-cyclodextrin. Most significant properties of cyclodextrins are that they can form solid inclusion complexes with wide range of guests (solids, liquids, and gases) in aqueous media and this inclusion formation is selective to the shape and sizes of the guests. Even in non-aqueous media inclusion complexes can also be obtained with co-solvent system. The stability of

these inclusion complexes depends on relative sizes of the host and guest molecules as well thermodynamic interactions between the cyclodextrins and guest molecule in the solvent system. The dissociation of these inclusion complexes are rather fast with increasing of water molecules surrounding environment (Connors., 1997; Gattuso et al., 1998; Rekharsky and Inoue., 1998; Saenger et al., 1998; Szejtli., 1998; Stella and He., 2008; Tabushi et al., 1984; Valle., 2004; Baker and Naguib., 2005).

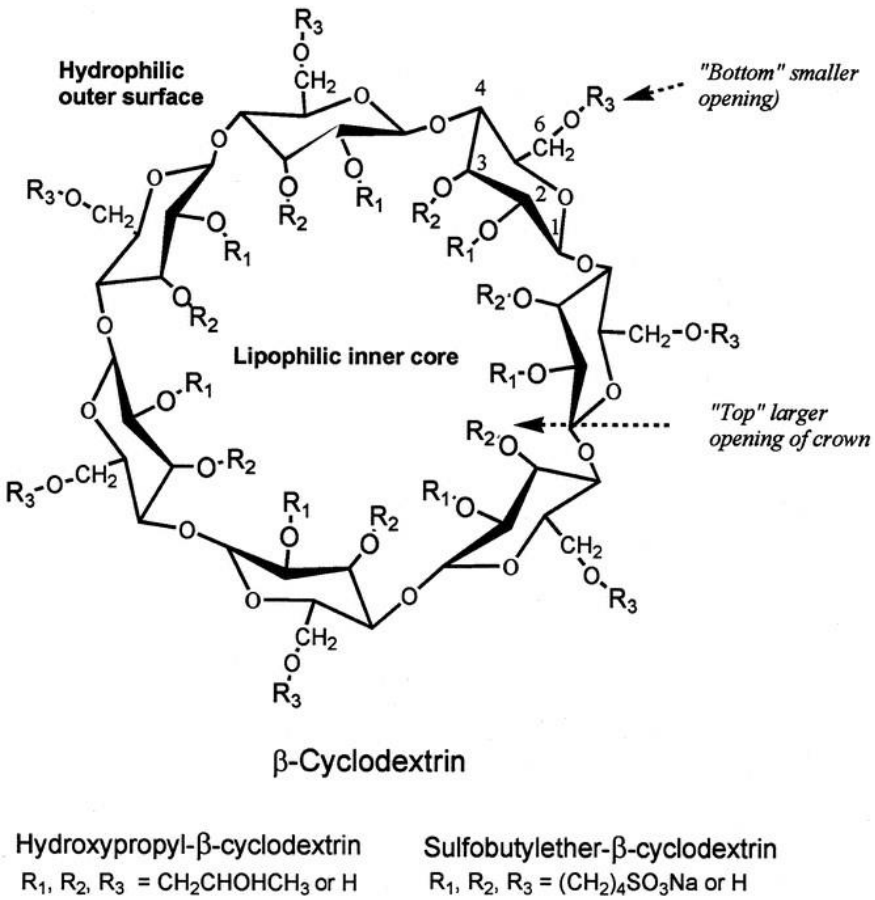


Figure 1: Chemical structure of β-cyclodextrin. Substituted functional groups (R1, R2 and R3) dictate the structural variation of cyclodextrins. Figure adopted from Baker and Naguib, 2005.

Cyclodextrins are widely used in pharmaceutical and biological applications. The complex formation properties of cyclodextrins are more attractive in formulating, as carriers and delivery systems with various drug and other active ingredients. Hence, cyclodextrins are used as potential drug delivery agents in numerous applications due to their ability to alter physical and chemical properties of guests in process of forming inclusion complexes. On the other hand, cyclodextrins increases the bioavailability of

drugs by firstly increasing their water solubility and then the stability of drug-CD inclusion complexes. So certain cyclodextrins are ideal candidates for drug formulations (Singh et al., 2002; Hirayama and Uekama., 1999; Loftsson and Duchene., 2007). The cyclodextrins are non-toxic and bioavailable and biodegradable, being allowed in various routes to administration to the body (Hedges., 1998). The other novel application of cyclodextrins is that they can be used as enzyme mimics due to their catalytic properties and in separation sciences. Apart from these, cyclodextrins are widely used in food industries, textile industries and cosmetics etc.. (Hirayama and Uekama.,1999; Szejtli., 1998; Hedges., 1998). Over these many advantages of cyclodextrins, however limitations in forming complexation with some drugs and in some cases their low stability and solubility have been found (Szejtli., 1984).

1.3.3 MOLECULARLY IMPRINTED POLYMERS

Molecular Imprinting is a simple and easy method in preparing receptor like structures in polymeric materials in tailor – made fashion. The most attractive phenomena in this process is self-assembly, which facilitates spatial arrangement of functional groups binding sites of host and guest in desired locations based on their molecular structures. The templates (guests) and functional monomers (hosts) interact with variety of non-covalent or covalent bonds. To perform imprinting procedure, sophisticated instrumentation is not necessary. The materials, solvents, experimental set up, and instruments are commonly cheap. The following templates removal from the imprinted materials yields template-specific cavities with binding sites at specific locations that promise recognition process. The corresponding imprinted receptor can recognize the same and/or structurally related template. The significant research has begun by introducing method and many of imprinted polymer materials are being used in various applications such as separation sciences, catalysis, bio-sensing and analytical devices etc.. (Dickey., 1949; Wulff et al., 1984; Kugimiya et al., 1998; Wulff and Knorr., 2002; Mosbach and Haupt., 1998; Mosbach., 1971; Mosbach., 1994; Shea et al., 1986; Takeuchi et al., 2001; Kugimiya et al., 1998; Sellergren., 1989; Whitecomb et al., 1995).

1.4 MOLECULAR IMPRINTING

Molecular Imprinting is a promising well-established supramolecular method for preparation of synthetic antibody like receptor structures in polymeric matrices, also known as molecularly imprinted polymers (MIPs). The technique is based on molecular recognition chemistry (host-guest chemistry or receptor-ligand recognition), which corresponds to lock and key model. This technique was firstly sparked in half of the 19th century by adsorbing different dyes on silica, later pioneered and developed since 1970's (Wulff et al.,) and 1980's (Mosbach et al.,). This technique has been matured and during past decades rapidly grown and well established in multiple disciplines areas to develop ideal, high sensitivity, high affinity

synthetic receptor materials, which are meanwhile thermally, physically, and chemically stable. MIPs have molecular memory and selectively recognize the target molecules with high binding affinities (Arshady and Mosbach., 1981; Wulff and Sarhan., 1972; Kaugimiya et al., 1995).

The syntheses of MIPs are simple, inexpensive, less laborious, and easy to performance for their molecular recognition. Therefore MIPs are considered as best alternatives to naturally occurring biological sensing elements. MIPs have been successfully employed in diverse applications as stationary phase in the separation sciences (Sellergren., 1990) and chromatography techniques (Haupt., 2010), analytical reagents in immunoassays (Muratsugu and Tada., 2013), artificial receptors for proteins (Matsui et al., 2009), drug screening, sensors (Kandimalla and Ju., 2004), and catalysis (Muratsugu and Tada., 2013).

In a typical imprinting procedure, the functional monomers and template molecules interacts with the reversible binding forms complexes (template-functional monomer complex), known as pre-polymerization complex. The interactions in the complexes are sufficiently enough to stabilize the equilibrium stage. MIPs are usually obtained by the polymerization of functional monomer-template complex with vast amount of crosslinking agents in presence of solvents (porogenic) resulted insoluble bulky crosslinking network polymers. Subsequently template extraction from the corresponding particles leaves the predetermined template specific cavities, termed as binding sites. These imprints possess complementary towards target templates physically (size and shape) and chemically (spatial arrangement of functional groups) (Ramstrom et al., 1994; Umpleby II et al., 2000).

Based on the nature of interactions in the complexes, molecular imprinting technique has been classified into three types: non-covalent, covalent, and semi-covalent imprinting. From the view point of material structure, imprinting methods have been categorized as imprinted organic polymers, inorganic (Sol-gel processed) and Sol-gel derived organic polymers (organic/inorganic hybrids). The typical imprinting procedure composed of the following 3 steps. Figure 2 illustrates schematic view of the typical imprinting procedure:

1. Preparation of template-functional monomer complex (non-covalently) or template coupled functional monomer adducts (covalently).
2. Polymerization and crosslinking of these prepared complexes or adducts
3. Template removal from the polymeric networks

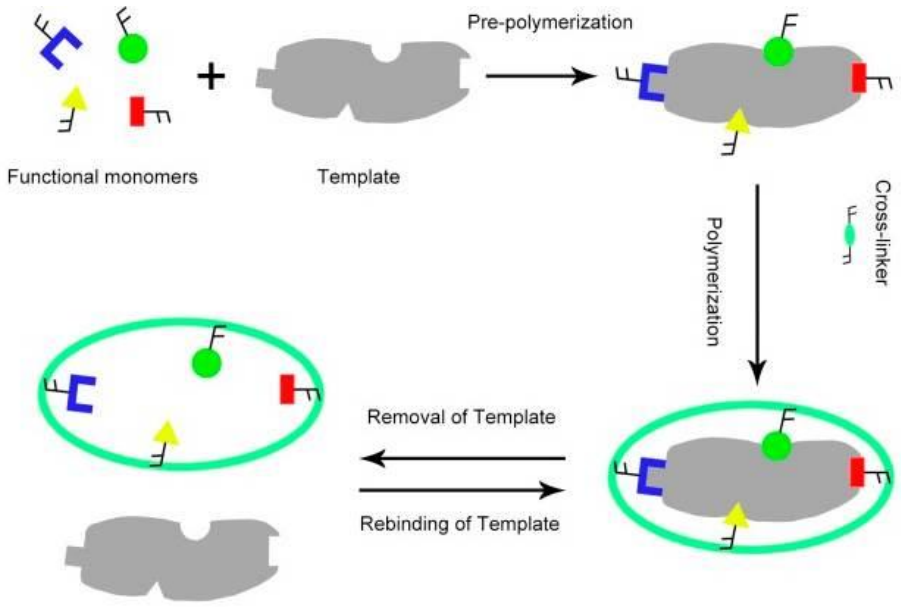


Figure 2: Schematic representation of typical imprinting procedure (Xu et al., 2015).

The major question that arises here is: do they maintain the same association and dissociation constants of the re-complexation between the template and MIP during recognition process and the template removal from the imprinted polymers? Such a question is raised not only in MIPs but also in all template-mediated synthetic receptors. To address this question, several factors should be considered. However; a simplified answer is no, since MIPs are obtained in bulky polymers; consequently binding sites are widely distributed throughout the polymeric networks leading to the heterogenic binding sites. To some extents, other several factors also influence this effect, like rigidity (degree of crosslinking), accessibility (size of the binding analyte and pores size on the polymers), imprinting and rebinding solvents (protic, aprotic, polar and non-polar), nature of interactions involved (non-covalent, covalent and semi-covalent), the nature of material used (organic, inorganic, or organic/inorganic hybrids) and type of imprinting procedure (epitope, adsorption and surface imprinting). Type of interactions in between the template and functional monomers also play an important role in explaining the homogeneity or heterogeneity binding sites.

1.4.1 NON-COVALENT IMPRINTING

Naturally occurring receptor-ligand or antigen-antibody interactions are non-covalent interactions. Molecular imprinting with non-covalent interactions can be performed easier than covalent ones, additionally template removal is rather easy and recognition

can be obtained due to that they are able to reform the same interactions with the binding sites of the imprinted polymers. Prior to polymerization, template molecule interacts with the functional monomers in self-sorting manner or *in situ* and forms template-functional monomer adducts in the solutions, also called as pre-polymerization complex. The wide variety of non-covalent interactions such as van der Waals forces, hydrogen bonding, electrostatic (ion-pairs, dipole-dipole) forces, and hydrophobic interactions etc.. Association constant of these adducts or complexes are low, consequently to establish equilibrium, excess of functional monomer are applied. Therefore, ratio of template and functional monomer turns to be an important parameter in imprinting method. Too much functional monomer added to shift the equilibrium constants may contribute to form high non-specific, heterogeneous binding sites, although too little functional monomer results poor yield of binding sites. The advantage of using non-covalent interactions: ease of preparation of template-functional monomer complex or adduct forms due to rather low bond energies, and widely available range of templates and commercial functional monomers. However, the limitation in this technique is that the use of excess amounts of functional monomer results in heterogeneity in the polymeric networks and moreover imprinting and rebinding solvents are limited. So, the ratios of functional monomers/templates are evaluated in practice by knowledge from literatures as well as trial and error fashion (Sallacan et al., 2002; Yan and Ramstrom, 2005; Ariga and Kunitake, 2006; Cram and Cram., 1974; Hioki and Still., 1998; Lin et al., 2006; Kirk et al., 2009).

1.4.1.1 Hydrogen bonding

In general, the wide range of non-covalent interactions can be employed meritoriously in imprinting method. Nevertheless, hydrogen bonding is most suitable in complex formation between template and functional monomer, recognition/rebindings due to their strength. The hydrogen bonds are formed between the hydrogen atom in the polar N-H or O-H bond and an electronegative oxygen or nitrogen atom, either intramolecularly, intermolecularly, or both. These are directional, reversible forming and short ranged forces; typically bond energy is 10-40 kj/mole and bond length is 1.9Å. Most commonly used carboxylic functional monomers such as methacrylic acid and acrylic acids forms hydrogen bonding with various templates as template-functional monomer adducts in aprotic solvents. Single or multiple functional monomers can be used in this imprinting method depending on template nature. Multiple hydrogen interactions lead to stable pre-polymerization complex, which contains high association constants and is sufficiently stable during polymerization. On the other hand, temperature also influences the association and dissociation constants of the complexes, strength of the hydrogen bond decreases while increasing the temperature. Subsequently, low temperature is usually employed to obtain strong complexes.

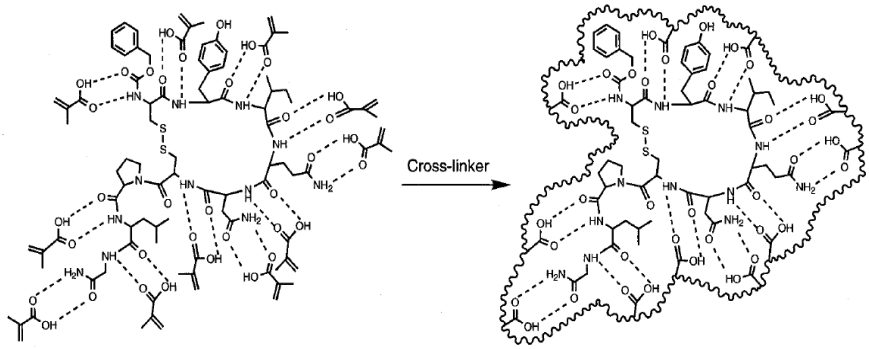


Figure 3: Schematic representation of self-assembly process of oxytocin peptide with methacrylic acid through hydrogen bonds and furthermore imprint (Kempe, 2000).

Template removal is rather easy in this method, polymer particles extracted with protic solvents such as water or methanol cleaves hydrogen bonds from the polymeric networks, thus resulted imprinted polymers are able to recognize the corresponding template or structurally template related compounds and forms hydrogen bonding in aprotic solvents. Figure 3 illustrates the oxytocin peptide forming hydrogen bonds with methacrylic acid in self-assembly process (Kempe, 2000). Wide range of templates was successfully imprinted in this approach including carbohydrates (Ramstrom and Lehn., 2000), proteins (Matsui et al., 2009; Janiak and Kofinas, 2007; Kempe., 2000; Hioki and Still., 1998; Lin et al., 2006: Hong et al., 1991), amino acids (Wu and Wang., 2008), lipids (Ogawa et al., 2012), nucleotide bases (Yano et al., 1998), and organic compounds (Kirk et al., 2009).

1.4.1.2 Electrostatic interactions

The electrostatic interactions are between and among the charged ions (cations and anions). Ion-ion interactions are long ranged and non-directional forces, bonding energies are inversely proportional to intermolecular distances and having comparatively higher bond energy than other range of non-covalent interactions. Due to their non-directionality these interactions are not well suited for the design of molecular recognition in imprinting methods. Typically bond energy is 60 kJ/mol. Ion-dipole forces are directional and weaker than the ion-ion but higher than dipole-dipole interactions, typically bond energy is 8 kJ/mol. These are well suited for the molecular recognition in imprinting methods owing to directionality and medium ranged bond energies. The dipole-dipole interactions are directional forces, too, forming either attractive or repulsion forces, which contains lower bond energies (typically 1 kJ/mol) and not well suitable in imprinting methods due to their weak bond energies (Yan and Ramstrom, 2005; Pietrzyk et al., 1957; Tabushi et al., 1984; Cram and Cram., 1974).

1.4.1.3 van der Waals forces

The van der Waals forces are found in all molecules includes sum of the attractive and repulsive forces among them. These interactions are weak and less specific compared to other range of non-covalent interactions which are described above. These forces are arised between the dipoles which were formed by unbalanced electronic distribution in neutral substances. Van der Waal's interactions are weak and short ranged forces, typically bond energies are 0.1-1 kJ/mol. These kinds of interactions are more effective while coordinating molecules which contain complementary shapes in the surfaces (lock and key concept) and important for host molecules to recognize the structure of the guest molecules. Van der Waal's interactions significantly contribute in molecular recognition, so it becomes attractive to be used in molecular imprinting and other supramolecular methods (Komiyama et al, 2003; Yan and Ramstrom, 2005; Ariga and Kunitake, 2006).

1.4.1.4 Hydrophobic interactions

Hydrophobic interactions arise among the hydrophobic species in aqueous medium and play an important role in self-assembly process. When hydrophobic molecules come in contact with water, hydrogen bonds disturbed in between the individual water molecules results in rearranging the water molecules through the hydrogen bond formation as hydration for layered structures around the hydrophobic species. The basic understanding on these interactions helps to minimize the contact of hydrophobic species to water and attractive strong interaction among them (Agira and Kunitake, 2006; Yan and Ramstrom, 2005).

1.4.2 COVALENT IMPRINTING

The template molecules are covalently linked to one or more functional sites of one or more functional groups and forms template-functional monomer adducts. The functional monomer of these adducts are then co-polymerized with massive amount of crosslinkers and additional polymerizable derivatives in the appropriate solutions resulted in bulky polymers. Further template removal and recognition or rebinding the analyte are carried out via specified chemical reactions. This method was firstly reported by Wulff and his coworkers. The key to success of this method is that using covalent linkages is able to form reversible reactions with the binding sites of imprinted polymers. Up to date, many of the covalent imprinting have been reported, such as boronic acid esters (Rajkumar et al. 2007; Lin et al. 2009), schiff bases (Wulff, Heide, and Helfmeier 1986), carbonate esters (Petcu et al. 2009), coordination bonds (Vidyasankar, Ru, and Arnold 1997), carbon-carbon double bonds (Kamplain and Bielawski 2006), cyclo additions (White et al. 2001), olefins (Cantrill et al. 2005), cyclic oligomers (Rowan, Reynolds, and Sanders 1999), trans-esterification (Fuchs et al. 2003), trans-acetalation (Mukawa et al. 2003), and disulfide bonds (Takeuchi et al. 2006; Takeda et al. 2009; Sellergren and Andersson 1990).

The advantages of covalent imprinting are stoichiometry of template and functional monomer in the adducts follows the 1:1 ratio, therefore no excessive amount of functional monomer needed resulted in the formation of homogenous binding sites throughout the polymeric matrices. Covalent interactions are strong enough, then stable polymerizable adducts are formed. As a result polymerization can be performed at high temperatures, under high power UV radiations or high or low pH, the choice of imprinting and rebinding solvents are free and other greater advantage of this method is that the binding sites are uniformly distributed and structurally as well functionally resembled one to another. However, some limitations in this method are i.) syntheses of template-functional monomer adducts are the biggest drawback and cost-effective; ii.) covalent bond energies are high and template removal is complicated, thus rather severe conditions need to be employed; iii.) available reversible forming covalent linkages, covalently bond forming templates and functional monomers are limited; v.) additional steps are needed to remove the reaction intermediates or by-products and guest binding and release are slow, if they involve in the formation and cleaving the covalent linkages (Mayes and Whitcombe, 2005; Lin et al. 2009; Kandimalla and Ju, 2004).

1.4.2.1 Imprinting with boronic acid esters

Covalent imprinting with boronate esters was firstly developed by Wulff et al., in 1977. The polymerizable conjugate synthesized from 4-vinylbenzene boronic acid (functional monomer) and 4-nitrophenyl-D-mannopyranoside (template). This derivative was co-polymerized with crosslinking agents (methyl methacrylate and ethylene dimethacrylate). The template was removed by cleaving the ester bonds (hydrolysis) from the polymers. Furthermore, resulted polymers successfully recognized the sugar molecules and bound strongly and selectively with binding sites of the imprinted MIP's. State-of-the-art many of the covalent imprinting with boronate esters were reported. Wulff et al., 1991 prepared galactose and fructose imprinted polymers with the boronate esters for racemic resolution of free sugars. The wide variety of templates have been imprinted by this approach for instance castasterone (Kugimiya et al., 1998) and nucleotides (Sallacan et al., 2002) etc.. The successful aspect with this approach is that these covalent linkages are able to form reversibly. To many extents, these boronate esters were incorporated in several applications such as fluroscent sensors, polyelectrolyte hydrogels, and chiral separations.

1.4.2.2 Imprinting with ketals and acetals

Ketones and aldehydes react with the diols forms ketals and acetals respectively. Shea et al., in1986, 1989, and 1991 introduced diol functional groups by using ketal conjugated diketone aromatic templates, which were subsequently co-polymerized. Template removal from the polymer particles yielded binding sites contained diols

which selectively recognized and rebound the original templates that equivalent to the vacant binding sites.

1.4.2.3 Imprinting with Schiffs bases

Schiff bases contain the carbon-nitrogen double bonded functional groups, are usually formed by condensing primary amine and a carbonyl group (typically an aldehyde). Schiff bases were successfully employed in imprinting methods to incorporate amine or aldehyde functional groups in imprinted polymers. Amino acid derivatives (Wulff et al., 1984) and 1,3,5-triazine (Sekar et al., 2011) derivatives were successfully imprinted by this method.

1.4.2.4 Imprinting with coordination bonds

The coordination bonds are direction specific and usually occur between the metal ions and electron rich atoms (supramolecular 2002 et al.,). Metal complexes (metal porphyrins) interacts with the template molecules through coordination bonds and these are adequately stable for covalent imprinting. These metalloporphyrins-template complexes are co-polymerized, upon removal of template obtained binding sites are exposed, which are able to re-form the same coordination bonds with the templates (Komiyama et al., 2003; Takeuchi et al., 2001). Metalloporphyrins-ligand complexes are involved in many of biological processes such as co-factors etc.. Takeuchi et al., 2001 prepared cinchonidine imprinted polymers with both vinyl substituted metalloporphyrins and MAA as a functional monomer and cinchonidine as a template molecule. Cobalt (II) ion complex, the aldolase-mimicking synthetic polymers, was able to catalyze the reaction between acetophenone and benzaldehyde selectively, which was usually entropically critical. However this cobalt mediated imprinted polymers contains benzaldehyde- and acetophenone-shape selective cavities and successfully catalyzes the reactions via C-C bond by aldol condensation (Matsui et al., 1996).

1.4.3 SEMI-COVALENT IMPRINTING

For balancing the great advantages and limitations of above described imprinting methods (covalent and non-covalent), a new kind of imprinting (semi-covalent) has been reported to subsidize the limitations by a combination of covalent and non-covalent strategy. During imprinting process, covalently linked template functional monomer adduct were co-polymerized, subsequently recognition was carried out non-covalently. This method has been firstly reported with L-tyrosin covalently bonded to methacryclic acid imprinted polymers, and subsequently the substrate selectivity was performed non-covalently (Sellergren and Anderson, 1990). Whitecomb et al., (1995) used covalently bonded template (cholesteryl (4-vinyl) phenyl carbonate ester) in imprinting process, then template was hydrolytically cleaved with the loss of carbon dioxide (CO_2). Resulted polymer particles bearing phenolic residue were able to

interact with the corresponding templates non-covalently through hydrogen bonding. The ester group (that was later on decarboxylated) has a dual role as linking template to functional monomer during polymerization as well as acting as a spacer between the recognizing templates and binding sites of the polymeric matrices. The carbonyl spacer was successfully used in imprinting methods such as urea derivatives (Lubke et al., 1998), nortriptyline receptors (Khasawneh et al., 2001) and estrone receptors (Ki et al., 2001). The other groups like salicylate or 2-hydroxy benzoate employed in making oligopeptide receptors (Klein et al., 1999), dimethyl silyl group (Kirsch et al., 2000) and silyl esters in heterocyclic aromatic receptors (Kirsch et al., 2004) have been successfully employed as a spacer groups in imprinting method. As recent development in these methods, disulfide templates were successfully imprinted as well (Mukawa et al., 2000; Takeuchi et al., 2002, Mukawa et al., 2006 and takeda et al., 2009).

1.5 IMPRINTING MATERIALS AND PROCEDURES

The recent developments in the molecular imprinting promoted developing intelligent materials with various functions and performances to recognize a broad range of molecules. Nowadays bio-molecule's recognitions have much drawn attention including proteins (Lin et al., 2006; Matsui et al., 2009; Kempe., 2000), saccharides (Ramstrom and Lehn., 2000), nucleic acids (DNA or RNA) (Yano et al., 1998), and lipids (Ogawa et al., 2012). During imprinting, whole of the template or part of the template (epitope imprinting), surface structure of molecule (surface imprinting), or immobilized imprinting can be applied. On demands during the recognition process, the functional groups in the binding sites will be removed via spacers or converted in to other form via post modification on MIPs (Takeuchi et al., 2002) and/or newly desired functional groups will be introduced. Molecularly Imprinted materials can be obtained as either organic materials, inorganic materials, or in combination between organic and inorganic species (hybrids). The typical imprinting process consists the following 4 steps: template-functional monomer adducts, copolymerization, template removal, and recognition process. Template-functional monomer adducts can be obtained either non-covalently or covalently, as described in details in the above section 1.4. The most commonly imprinting materials and procedures are classified in to main three main categories as described below:

1. Organically imprinted polymers (Polymerization)
2. Inorganically imprinted polymers (Sol-gel processing)
3. Inorganic/organic Hybrids (Sol-gel derived organic hybrids)

1.5.1 ORGANICALLY IMPRINTED POLYMERS

Naturally occurring bio-macromolecules such as polysaccharides, proteins and nucleic acids composed with the monomeric units of simple sugars are connected with the acetal linkage, amino acids are connected with amide bonds, and nucleotides are

connected with phosphodiester bonds respectively (Fox and Whitesell, 2004). Polymers can be composed of either one type repeating subunit (homopolymers) or mixture of subunits (copolymers). Polymerization can be obtained either by radical or ionic (cationic or anionic) vinyl polymerization or step reaction polycondensation methods (Komiyama et al., 2003; Diederich and Stang, 2005). In this PhD study, free radical polymerization has been performed to prepare the imprinted polymer matrix. All the materials and chemicals needed for organic based imprints are functional monomers, templates, crosslinkers, initiators, solvents, co-solvents, and porogens.

1.5.1.1 Functional monomers

During imprinting process, functional monomer interacts with the template molecules either covalently or non-covalently. In a non-covalent imprinting, numerous functional monomers are commercially available and are readily form hydrogen bonding, electrostatic interactions, and hydrophobic interactions with their corresponding template molecules. The choice and the amount of the functional monomers must be carefully evaluated, that depends on the nature and binding sites of functional groups on template and choice of the interactions. The most commonly used functional monomers in non-covalent imprinting are methacrylic acid (MAA) and vinyl benzene (Komiyama et al., 2003; Sellergren et al., 2006). On the other hand, the various functional monomers are available for the covalent imprinting; however, it should form covalent linkage to the corresponding templates. 4-vinyl phenyl boronate (Wulff and Sarhan., 1972) has been most frequently employed. The functional monomers with the disulfide linkages to templates are attractive ones for the use in covalent imprinting. In current PhD study, Prop-2-ene-1-thiol has been chosen as a functional monomer since it's able to form disulfide bond with various templates such as benzyl mercaptan, amino acid (Cysteine), and proteins which contain at least one cysteine moiety in its sequence.

1.5.1.2. Crosslinkers

Molecular imprinting is a template mediate co-polymerized method. Once template-functional monomer adducts are formed, they are co-polymerized with crosslinkers. Usually divinyl group contained crosslinkers are employed and amount of crosslinkers dictates the density and rigidity of the resulted network polymer. Adding large amount results in the dense polymers and small amounts leads to loosen and open structured polymers. These both situations have advantages as well disadvantages. For dense polymers, binding site cavities are very close towards the corresponding template moieties that contributes highly specific, selective recognition. On the other hand, it limits the accessibility of binding sites and diffusion of template molecules (Jie and Xiwen., 1999; Zhang et al., 2001; (Komiyama et al., 2003).

The polymers with open structured (loosen network) yield low specific binding sites, because after template removal polymeric networks might shrink or elaborate, and therefore might change the binding cavities. So the molar ratios of crosslinkers and functional monomers need to be carefully chosen for obtaining ideal polymers. The wide varieties of commercially available crosslinkers are available and are miscible in organic solvents. The most commonly used crosslinkers in organic solvents are with ethyleneglycol dimethacrylate (EGDMA), divinyl benzene, and trimethylopropane trimethacrylate (TRIM). When imprinting is performed in in polar solvents, N, N'-methylenebisacrylamide as a crooslinker will be employed due to it solubility in water (Sellergren, 2001; Komiyama et al., 2003; Stevens, 1999).

1.5.1.3 Solvents

Solvents play a crucial role in imprinting procedure. During imprinting process, solvents occupy spaces in between the networks, and leave the product a porous structure. The imprinting conceded absence of solvents results too dense and rigid polymers, accordingly too hard binding sites will be formed. During molecular recognition guest binding and release also depends on the porosity of the polymeric network, so solvents should provide porous structure on the polymer surfaces, which plays a crucial role in recognition. Ideally, solvent should solubilize all components of the reaction as well provide porous structures in polymers and should not interact or interfere in reaction progress. In general, during polymerization, heat is generated, so these solvents conduct the generated heat and suppress the unwanted side reactions (Komiyama et al., 2003).

The choice of the solvents also depends on the type of interactions used for the imprinting, for instance decision on the solvent for non-covalent imprinting is more critical, protic solvents such as MeOH and EtOH suppress the hydrogen bonding efficiency during template-functional monomer adduct formation as well in recognition process. On the other hand, these solvents are best suit for extracting the templates from the polymeric networks. Chloroform, acetonitrile (ACN) and dimethylsulfoxide (DMSO) are most commonly employed solvents in non-covalent imprinting (Sellergren et al., 1988; Asanuma et al., 2000). In covalent imprinting the choice of solvents are broad until it solubilizes all components in the reaction mixture. Sometimes co-solvent(s) or porogene(s) are employed to form homogenous reaction mixtures and forms porous structure in the MIPs. The most commonly used co-solvents or porogens are tetrahydrofuran (THF), NN'-dimethylformamide (DMF) and toluene (Komiyama et al., 2003; Sellergren et al., 2006; Yu and Mosbach, 1997; Sellergren et al., 1988; Mayes et al., 1994).

1.5.1.4 Polymerization procedure

Polymerization can be performed via free radical or ionic reactions on vinyl functional monomers, while free radical polymerization is most commonly employed. Three

main steps are involved in this method: initiation; propagation; and termination (Fox and whitesell, 2004). The polymerization starts with the decomposition of the initiators for formation of free radicals, most commonly employed initiator in imprinting procedure is 2,2'-azo-bis(isobutyronitrile) (AIBN) or 2,2'-azobis(2,4-dimethylvaleronitrile) (ADVN). The initiator can decompose either photochemically or thermally. The fast decomposition intensifies the free radicals that lead to fast polymerization. When early termination and/or chain transfer reactions takes place, consequently low molecular weighted polymers are formed. Usually at lower temperatures and longer times are more suitable conditions for non-covalent imprinting performed under UV irradiation at 366 nm, because either hydrogen bonding or other non-covalent forces in between template-functional monomer adducts is higher temperature unfavorable. Photochemically initiated polymers are able to recognize templates from the structurally related molecules at ambient temperature conditions, but thermally initiated polymers recognize the template at elevated temperatures (Sellergren., 2001; Kemp and Mosbach., 1995).

1.5.2 INORGANICALLY IMPRINTED POLYMERS

The fabrication of materials and devices from glass and ceramics is an attractive subject in fast growing materials science and has great potential applications in wide areas including biosensors, bioelectronics, catalysis, and drug delivery. The porous materials are classified in to three main classes depending on the pore sizes, namely microporous (< 2.0 nm pore size), mesoporous (2.0 – 50.0 nm pore size) and macroporous (>50.0 nm pore size) (Sing et al., 1985). Sol-gel particles are extensively used in molecular recognition, bio-encapsulation and delivery tools (DNA, RNA, Proteins and drugs etc.) due to their uniformity in physical properties and their tunable nano scale sizes. Nano particles can be achieved through the sol-gel process of metals or metal alkoxides (Sing et al., 1985; Pierre., 1998).

A colloid is a suspension, dispersed phase in range from 1-1000 nm, driven by negligible gravitational forces and short range forces such as van der Waals forces and surface charges. A sol is a stable colloidal suspension of solid particles (2 -200 nm) in a liquid and a gel is three dimensionally connected solid network which expanded throughout liquid medium uniformly. Aerosol is a suspension of colloidal particles in gas, while emulsion is a suspension of droplets in another liquid medium. Sol-gel process is a solution process to prepare inorganic materials with high purity and homogeneity. The whole sol-gel process is orderly progressed by following steps: forming a solution, gelation, aging, drying and densification, during this process system state is transits from one (liquid or sol) to other (solid or gel) (Brinker and Scherer, 1990).

The diverse applications of these inorganic materials were widely used in molecular imprinting. The most commonly used precursors for sol-gel processing are inorganic salts (Al $(NO_3)_3$ or metal alkoxides (TEOS). The solvent evaporated from the sol-gel

processed particles at the ambient pressure results the xerogel (dry gel), during this process gel may crack due to the shrinking in the network. Hence careful evaporation is needed to obtain aerogel. The imprintable templates will be coupled into the functional monomers through self-assembly or covalently bonds. The chemicals and conditions which used in this process such as concentration of sol-gel precursors (functional monomers), catalysis (acid or base), temperature, water content, solvents and co-solvents etc. will greatly influence the properties of imprinted materials and imprinting effect (Brinker and Scherer, 1990; Pierre., 1998; Diederich and Stang, 2005).

1.5.2.1 Sol-gel precursors

The wide range of metals or metal alkoxides is used as precursor molecules for the sol-gel imprinting. Metal alkoxides are members of the metalorganic compounds group, where organic ligand attached to a metal or metalloid. One most extensively studied precursor molecule is tetraethoxyorthosilicate (TEOS). Metal alkoxides undergoes hydrolysis because they can readily react with water. This hydrolysis may be partial or in completion. Partially hydrolyzed molecules are able to link each other via condensation reaction. The reaction proceeds through series of condensation reactions forming highly crosslinked Si-O-Si bonded three dimensional network structures, as formulae represented in equation 1.1, 1.2, and 1.3, describing the hydrolysis and condensation of sol-gel process.

$$\equiv Si-OR + H_2O \underset{ether\,formation}{\overset{hydrolysis}{\rightleftharpoons}} \equiv Si-OH + ROH \quad \textbf{(Eq. 1.1)}$$

$$\equiv Si-OR + HO-Si\equiv \underset{alcoholysis}{\overset{alcoholcondensation}{\rightleftharpoons}} \equiv Si-O-Si\equiv + ROH \quad \textbf{(Eq. 1.2)}$$

$$\equiv Si-OH + HO-Si\equiv \underset{hydrolysis}{\overset{watercondensation}{\rightleftharpoons}} \equiv Si-O-Si\equiv + H_2O \quad \textbf{(Eq. 1.3)}$$

Equation 1.1, 1.2, and 1.3 describing the hydrolysis and condensation of sol-gel process, R is an alkyl group (Pierre., 1998).

The wide variety of precursor molecules, were employed in imprinting for diverse templates such as TEOS for methanol and cresol (Pinel et al., 1997) and phenyltrimethoxysilane (PTMOS), methyltrimethoxysilane (MTMOS), and TMOS for dopamine (Makote and Collinson, 1998) etc..

1.5.2.2 Catalysis

The sol-gel process of the metalalkoxides is a slow reaction, which can be catalyzed by either acid or base (Yan and Ramstrom, 2005). The concentrations of catalyst make great impact on reaction proceedure and resulted polymers. Sol-gel process proceeds at low pH 2.5 in aqueous solution with initiated hydrolysis due to the high concentration of protons. Under the acidic conditions hydronium ion and in basic condition hydroxide ion will attack to oxygen on one of alkoxide group, reaction occurs via bimolecular nucleophilic substitution (S_N2) as illustrated in Figure 4 showing the acid and base catalyzed procedure.

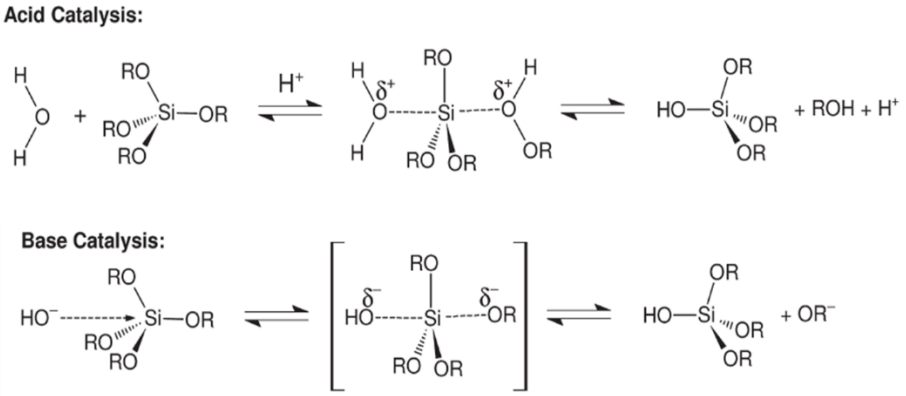


Figure 4: Acid/base catalyzed hydrolysis of a silicon alkoxide (Kickelbick., 2007).

Under the acidic condition, hydrolysis goes relatively faster compared to condensation, this results in highly branched silica network and clear gels with polymer-like structures (Yam and Ramstrom, 2005). Nevertheless, under alkaline solutions, condensation is relatively faster than hydrolysis, leading to the formation of dense silica particles. Final size of the spheres depends on the initial concentration of water and base. Usually mineral acids, ammonia, alkali metal hydroxides and fluoride ions are employed as catalysts in sol-gel system (Novak., 1993; Kickelbick., 2007; Yam and Ramstrom., 2005; Hench and West., 1990).

1.5.2.3 Solvents and co-solvents

The sol-gel precursors are typically immiscible with water, but water is needed to perform sol-gel process; therefore, another solvent (co-solvent) should be added. The nature of solvent and concentration typically influence the final product. Many solvents have been employed in sol-gel process. The choice of solvent also finds the polarity and water solubility of the solvents to solvate polar, tetra functional species during the process. However, for imprinting in the sol-gel matrices, the solvent must be carefully chosen based on type of interactions that had been applying in the

imprinting. For example, for hydrogen bonds applied in the imprinting, the aprotic solvents are the best choices.

Ethanol is a most commonly used solvent in sol-gel processing due to its best solvating the sol-gel precursors, especially TEOS. While ethanol is, at the same time, a by-product in this type of processing, which also participates in an etherification or alcoholysis during the reactions, these are the reversible reactions of equation 1.2 and 1.3 (Novak., 1993; Yam and Ramstrom., 2005; Kickelbick., 2007; Hench and West., 1990).

1.5.2.4 R-Value

The molar ratio of water and silica precursor (H_2O: Si) is called R value. R value should be chosen carefully because water is a by-product in reaction. Typically, R value is set from less than 1 to over 50, depending on the desired end product. Theoretically R value of 2 is sufficiently needed to complete hydrolysis and condensation obtaining anhydrous silica, nevertheless in great excess of water, the reaction does not complete (Brinker and Scherer, 1990). The reaction under acid catalysis, increasing the water content accelerates the hydrolysis. Under base catalyzed reaction, large R value promotes the siloxane bond hydrolysis. On the other hand, an increasing R value results in the liquid-liquid immiscibility, unless alcohol is produced as a by-product from the reaction leading to homogenization in the final product (Brinker and Scherer, 1990; Kickelbick., 2007).

1.5.3 ORGANIC/INORGANIC HYBRID MATERIALS

In above section 3.1 and 3.2, we described the type of materials and synthetic methodologies for imprinting with organic and inorganic networks respectively. The target molecules are in angstrom size, recognition can be obtained in organically imprinted matrices in presence of organic media, but naturally occurring biologically active nanometer scaled large targets such as peptides, ligands, vitamins, hormones and antibiotics can be recognized in plasma media (inorganic or water) by natural receptors. So, these imprinted organic polymers are not sufficiently needed to recognize these targets in water because of most non-covalent interactions such as hydrogen bonding used in process will be destroyed by water, addition to this, organic networks significantly swell in solvents and are not thermal stable for certain type of applications. Imprinted inorganic matrices shows highly crosslinked silica network and best suitable for recognition of nanometer ranged templates in hydrophilic systems. Meanwhile organic polymers have flexibility, low density and formability, but inorganic glass or ceramics have excellent mechanical and optical properties. The combination of inorganic and organic phases within the materials leads to hybrid materials, which inherits both advantages of glass and organic polymers such as high mechanical strength, good transparency, chemical inertness, high stability and negligible swelling in aqueous and organic solvents. Sol-gel reaction processed at

lower temperatures is the most attractive feature to incorporate the organic polymers in to the silica matrix without decomposition (Ogoshi and Chujo, 2003, Schottner., 2001; Chung et al., 2005).

Hybrid materials contains hydrophobic (organic) and hydrophilic (inorganic) properties, so based on these combined results, it can be used in amphiphilic systems. Numerous ways may incorporate organic content in to inorganic networks. Hybrid materials can divided into two main categories: (1) homogenous system which derived from miscible organic and inorganic phases; (2) heterogeneous system derived from separated domains. Furthermore hybrid materials had been classified in to five major types based on their structures and phase connectives (Novak 1993).

1.5.3.1 Imprinting with Organic- inorganic hybrid materials

The versatile uses of hybrid materials are more attractive in the molecular imprinting. During the past decades imprinted organic-inorganic hybrid materials have been successfully employed in wide range of fields including organometalic chemistry, catalysis, supramolecular chemistry, separation sciences (column preparation) and sensory applications etc.. The major motive force behind these developments with these materials, is that they contain new and different properties which are not found in traditional macroscale and microscale materials. Most of the organic/inorganic hybrid materials are in nanometer range, typically 1-100nm. Based on the structural difference, hybrid materials are divided into two main classes. Class I, hybrids contains weak interactions such as hydrogen bonds or van der Waals forces or electrostatic interaction in between the organic and inorganic phases. Class II, hybrids contains organic and inorganic phases are connected through strong covalent interactions (Ogoshi and Chujo., 2003, Chung et al., 2005; Norrlow et al., 1984; Jain et al., 2005; Schottner., 2001).

1.5.3.2 Pathways to imprinted hybrids

The most important aspect in the imprinting is to introduce the template in the hybrid materials. In general, template molecules interact with the functional monomers (either organic or inorganic or both) covalently or non-covalently, so, these interaction makes template molecules soluble and template should be introduced prior to the polymerization.

Molecularly imprinted hybrid materials can be obtained in three different approaches. In the first approach, prior to the polymerization all the ingredients of the imprinting are dissolved in the solvent or solvent systems to make homogenize mixture, there is no interaction between the organic and inorganic precursor molecules. Sol-gel reaction initiated in the presence of acid or base catalysis and subsequently radical polymerization initiated upon reaction mixture reached gelation time (Pope et al., 1989). The resulted hybrid materials contains weak interactions between organic and

inorganic networks as class I hybrids which was described above. In the second approach, organic and inorganic precursor molecules interacts with the covalent linkages and prior to the polymerization all ingredients used in the imprinting are mixed thoroughly. Sol-gel process initiated and subsequent organic polymerization yields the imprinted hybrid materials (Class II), two phases are connected with covalent linkages.

In the third approach, prior to the polymerization all reaction ingredients are mixed in solvent systems and covalently linked organic and inorganic precursors will be used. During the polymerization, sol-gel process and organic polymerization simultaneously initiated. So, organic and inorganic phases connected with covalently and mutually interpenetrated in the resulted imprinted hybrid materials (new class of hybrids or class III hybrid) (Novak 1993). In this PhD study, class I hybrid materials were prepared. Besides these, other methods are also available to prepare hybrid materials, comprising post-synthesis like grafting or direct co-condensation of tetraalkoxysiloxane and one or more oraganoalkoxysilanes (Stein et al., 2000 and Morey et al., 1995). These methods permit the modification of the synthesized silicates surfaces and alter the surface reactivity. The desired functional groups will be introduced with these methods, for instance in post-synthesis grafting method, organosilane coupling agents reacted with the surfaces of the prefabricated inorganic materials and forms surface silanole groups (Stein et al., 2000), (Feng et al., 1997) and (Mercier et al., 1997). To major extent, this modification or introduction of new functional groups of the silicate surfaces are to be used in the grafting or immobilze the bio-macromolecules or bioencapsulation (Gill and Ballesteros., 2000; Diaz and Balkus., 1996; Jain et al., 2005; Norrlow et al., 1984).

1.6 APPLICATIONS OF MOLECULAR IMPRINTED POLYMERS

During past decades, more advanced techniques have been introduced to analyze the compounds, complex structures, selective recognition with high affinities for improving them in reliability and simplicity. Recent advances in developing analytical methods are having more attractive applications in wide variety of areas. A numerous biological recognition systems are being exploited, however biological recognition agents such as antibodies, enzymes and other ligands are very specific, sensitive towards receptors, and available binding sites with very low densities. The molecular imprinting technique are emerged as best alternative to natural ones having mimicking them in structurally and functionally. Molecularly imprinted polymers (MIP's) are synthetic polymers which contain predetermined selective cavities towards analytes and structurally related templates. These are obtained from polymerization of template-functional monomer adducts co-polymerized with crosslinkers in appropriate solvents, further templates removal from polymeric materials results in template specific cavities. This method is very promising to prepare mimicking synthetic receptor like structures in polymeric matrices with a robust nature. Preparations of MIP's are simple, less laborious and inexpensive.

MIP's are applied in diverse applications including separation science (sellergren et al., 2001), analytical science (piletsky and Turner et al., 2002; Haupt and Mosbach, 2000, Janiak and Kofinas, 2007; Jenkins et al., 2001), synthetic receptors (Allender, 2005; Ansell, 2001; Haupt, 2003; Batra and Shea, 2003; Lettau et al., 2004; Kempe, 2000), drug screening, sensors, catalysis, and immunoassays etc..

MIP's have been successfully used in chromatography applications for separation and purification of different pharmaceutical products. In general, pharmaceutical sector demands for separation of enantiomerically pure compounds, especially mixtures of racemates (Kempe and Mosbach, 1995). MIP polymers are attractive ones using chromatographic and solid phase extraction (SPE), due to their having inherent stability, inexpensive, less laborious, and simple experimental settings. So, MIP's materials are used as stationary phase in chromatographic columns includes HPLC and capillary and membrane electrophorosis systems (Schweitz et al., 1998) and used in SPE systems (Sellergren, 1999; Zue et al., 2002). To extend this, significantly MIP's have showed high stereo selectivity and enantiomer selectivity towards wide range of compounds includes derivatives of amino acids (Sellergren et al., 1994; Rajkumar et al., 2007), proteins (Ramstrom et al., 1994; Fishcher et al., 1991; Janiak and Kofinas, 2007) and other biological active agents (Kempe and Mosbach 1994). Membrane based chemical separations with ultrathin films of MIP's were employed. These thin membrane films demonstrate high selectivity and separation for the target compound (Hong et al., 1998). The imprinted polymers illustrate reasonable strength and binding selectivity towards the analytes, which are comparable to the recognition of those antibody-antigen, enzyme–substrate and ligand receptor interactions. These could be more attractive in developing synthetic antibody-like receptors, which can be serves as antibody binding mimics also called plastic antibodies. The rational design and synthetic routes of plastic antibodies are rapid and more efficient. These synthetic receptors are in robust nature for attractive applications in using bioanalytical devices.

1.7 IMPRINTING WITH DISULFIDE BONDS

Disulfide bonds (-S-S-) are covalent bonds, formed by oxidation of two thiol groups (–SH). Naturally occurring cysteine amino acids constitute thiol functional group at the side chain and are able to interact with other cysteine amino acid containing protein molecules via disulfide linkage and facilitate the formation of three dimensional structural proteins also known as protein folding (native state). Proteins are active towards biological functions in native state. In the above sections, we have described the advantages and limitations of the both covalent and non-covalent imprinting. To overcome some of the limitations, disulfide based imprinting has been introduced (Mukawa et al., 2000). The attractive feature of these disulfides are that bond energies are typically lower than that of covalent linkages (C-C or C-H bond) and higher than non-covalent ones (hydrogen or electrostatic forces). Disulfides can be used in molecular imprinting due to their availability in many of templates

including small organic molecules as well as bio-macromolecules (proteins), combined with the easy removal of templates by reduction and flexibility for modifying the binding sites for desired recognition targets with high binding constants either by non-covalently or covalently.

Up to date few of the researchers reported usage of the disulfide bonds in the imprinting techniques. However, recognition were carried out non-covalently (Semi-covalent Imprinting). In this present PhD study, we developed a novel method for using disulfide bonds in molecular imprinting and recognition process. The attractive aspect of this method is that the imprinted small organic molecules were introduced with tunable binding sites and recognition can be carried out at all atmospheric conditions in protic and aprotic solvents. In a typical imprinting methodology with disulfides consists of four steps, 1.) Template–functional monomer adduct; 2.) Reduction of disulfides; 3.) Post-imprinting modifications; 4.) Recognition by reforming disulfides. The typical imprinting procedure with the disulfide bonds is schematically shown in Figure 5.

Oxidation of two thiol functional groups (R-SH) forms disulfide bonds in symmetrical compounds (R-S-S-R). Oxidation can occur in air, iodine, peroxides, or other oxidative agents. While obtaining disulfide bonds in unsymmetrical compounds, thiol functional groups (R-SH) acting as a nucleophile attack the disulfide compounds (R^1-S-S-R^2) and form new disulfide linkages (R^1-S-S-R or R-S-S-R^2). A number of synthetic routes have been reported to obtain unsymmetrical disulfide compounds. The polymerizable adducts were prepared by fusing polymerizable precursor (either vinyl functionality or with sol-gel precursor) to template molecules through disulfide bridge. Various synthetic routes have been reported for preparing polymerizable adducts with disulfides (Mukawa et al., 2003; Takeda., 2009; Takeuchi et al., 2006). Figure 5 shows that a disulfide bridge is connecting a template and a functional monomer.

In this PhD work, we have designed and synthesized wide range of template molecules includes phenolic compounds, cysteine, and synthetic oligopeptides. The incorporated polymerizable precursor molecules were also assessed and synthetized for diverse purposes, which includes vinyl functional groups and sol-gel precursor molecules, the reduction of disulfide bonds from the imprinted materials resulted in tailor-made template specific cavities. In general, disulfide bonds are easily cleavable with certain reducing agents. In biological context, disulfides play an important role in protein folding and function, so reduction of disulfides helps opening folded protein structures and inactive state. Oxidation of numerous synthetic methodologies reported for the disulfide formation is simply by oxidation of thiol groups and easily cleavable by reducing agents such as sodium borohydarte ($NaBH_4$), lithium aluminum hydride ($LiAlH_4$), or sodium hydride or dithioltritile (DTT). Since this disulfide cleavage is simple, such process gains much attention in robust polymeric materials. The cleaved disulfides leave tailing free thiol containing template specific cavities in the polymeric

networks. (Zhang et al., 2011; Zubarev et al., 1999; Greytak et al., 2001; Mukawa et al., 2003; Takeda., 2009; Takeuchi et al., 2006). We have tested different reducing agents (DTT, $NaBH_4$, $LiAlH_4$, and NaH), and diverse methodologies.

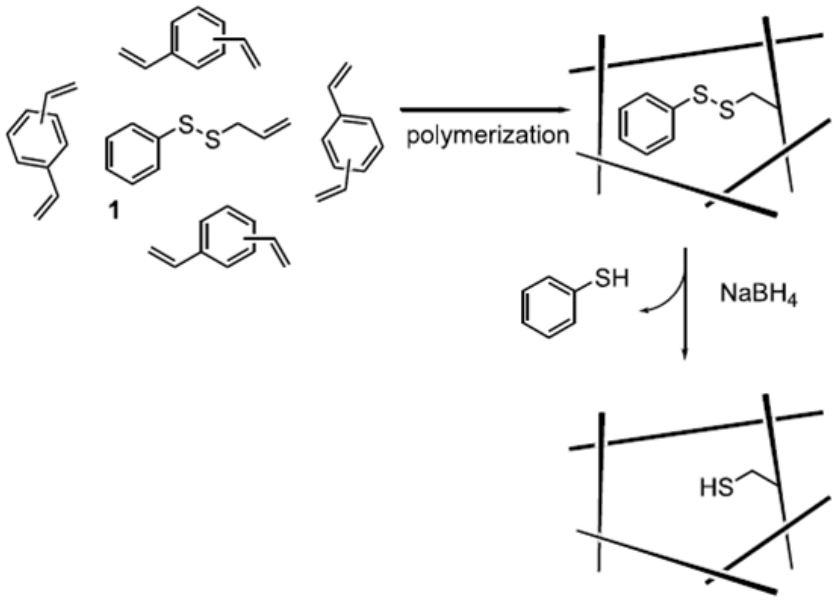


Figure 5: Schematic view of typical imprinting procedure using allyl phenyl disulfide (1) as template and co-polymerized with divinylbenzene. After polymerization, disulfides were reductively cleaved with sodium borhydrate (NaBH4), and resulted in thiol group containing binding sites which cavities are complementary to the thiophenol or other structurally similar molecules.

The imprinted polymers, which contained thiol-"bonding" sites, are attractive to recognize various templates and structurally related templates via covalently forming disulfides or non-covalently hydrogen bonding or other bindings. The proof-of-concept thiol-disulfide exchange reactions are very useful in recognizing the templates with disulfide components. To facilitate the thiol-disulfide exchange recognition, the imprinting polymer bonding sites are modified (post-imprinting modified) for bonding ability, sulfhydryl groups in binding sites were modified into sulfonic acids and integrated with other cofactors. (Takeda et al., 2009; Takeuchi et al., 2006). In this study, we introduced the method converting thiol groups into thiolated form, these results in increasingly nucleophilic nature in binding sites which are able to recognize templates and structurally related template having disulfide in their structure. This is the one of the key concept in recognizing the peptides.

CHAPTER 2. BENZYL MERCAPTAN (BMT) SYNTHETIC RECEPTORS IN ORGANIC POLYMERS

2.1 MOTIVATION

As described in Chapter-1, molecular imprinting methodology is a promising method in preparing man-made mimics of synthetic anti-body like receptor structures. These imprinted polymers have greater advantages and be open for scientists to design novel intelligent materials towards wide variety of templates which can include chemical compounds and biologically important amino acids, carbohydrates, lipids, nucleotides, and proteins. The templates and functional monomers can form complexes via various covalent and non-covalent interactions in tailor-made fashion. Covalent and non-covalent molecular imprinting presents their own advantages and limitations (as described in Chapter-1). We have started evaluation on both these two methodologies and found that covalent rebinding from covalently imprinted polymers may dramatically improve the selectivity of rebinding, when comparing with non- and semi-covalent imprinting. The choice of covalent bonds falls in significant importance in template removal and recognition process: whether or not such type of bonds can be easily cleavable under mild conditions and can be formed reversibly with templates during recognition process. The additional advantage with disulfides is that polymerization can be performed at relatively high temperatures or under powerful ultraviolet irradiation.

With this preliminary evaluation we found attractive features in disulfide bond based covalent imprinting. Disulfide bond energies are comparatively lower than other common covalent linkages but higher than non-covalent interactions. These bonds can be used in molecular imprinting due to their availability in many templates. The disulfide bonds can be cleaved reductively and templates can be recognized by reversibly formed bonding nature of disulfide (thiol-disulfide exchange/tethering strategy). The attractive feature of thiol containing binding sites of polymers can be the chemical modification without altering physical properties. Up to date few researchers have reported disulfide based covalent imprinting (Greytak et al., 2001; Mukawa et al., 2003; Takeda., 2009; and Takeuchi et al., 2006). As assay studies in this work, small organic compounds have been imprinted with disulfide bonds and recognized both covalently and non-covalently with the post-modified MIPs and NIPs.

In this part of the PhD study, we have designed a novel post-modification process to convert thiol containing binding sites in imprinted polymers to thiolate form in order

to facilitate reversible forming of disulfide linkage with templates through thiol-disulfide exchange strategy. With this modification; the significant change in efficient recognition and high binding capacities has been observed. To the best of our knowledge, this could be the first example of using disulfide based covalent imprinting and covalent recognition.

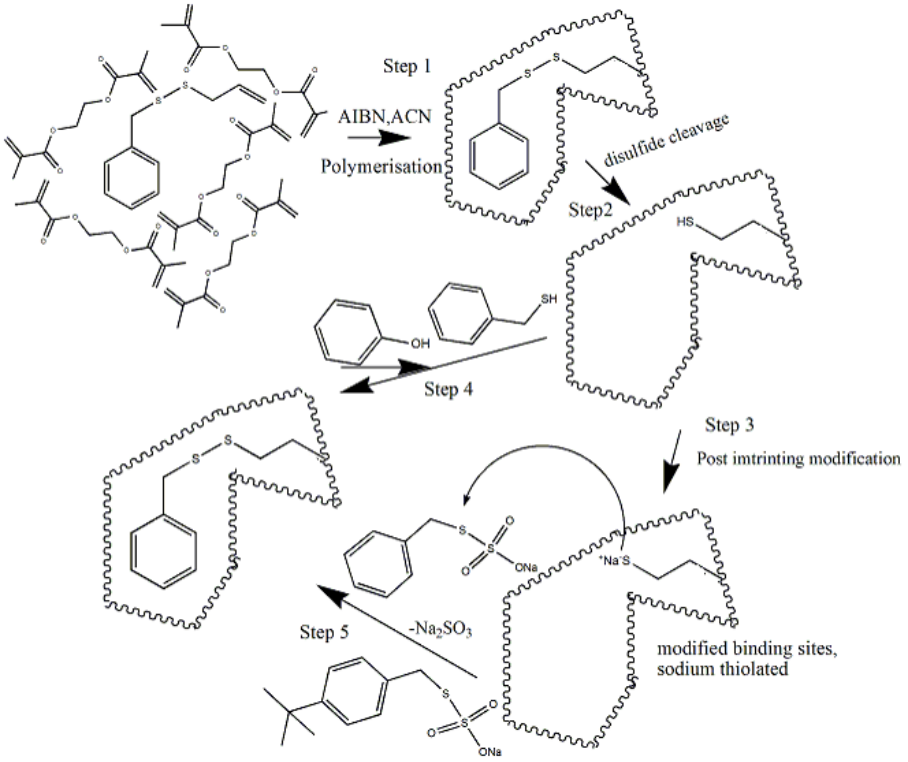


Figure 6: Schematic representation of molecular imprinting and recognition of benzyl mercaptan: step 1: Polymerization; step 2: template removal by cleaving disulfide bond; step 3: recognition through post modification on MIPs (and NIPs); and step 4: recognition. (Burri and Yu., 2016).

We have started imprinting small organic compounds. A benzyl mercaptan imprinted synthetic receptor was prepared via covalent imprinting of 3-benzyldisulfanylprop-1-ene which serves used as a template-functional monomer adduct. This functional monomer contains a template and a vinyl moiety for polymerization/crosslinking with 1:1 molar ratio, these were expected to increase the forming homogenous binding sites (Andersson et al., 1999; Katz and Davis., 1999; Whitcombe et al., 1995; Sellergren and Andersson 1990). Functional monomers were co-polymerized/crosslinked with ethyleneglycol dimethacrylate (EGDMA) under UV radiation at ambient and heat at 80ºC. The control polymers were prepared by means of thermal crosslinking with

prop-2-ene-1-thiol and EGDMA. Figure 6 presents the schematic view of imprinting and recognition of benzyl mercaptan.

Further disulfide bonds were reduced, yielded binding sites with the surface tailing thiol groups (-SH). Subsequently these binding sites were converted in to sodium thiolate with the novel post imprinting modification. These modified binding sites serve as covalent binding sites, making template molecular recognition available (Takeuchi et al. 2006). These covalently imprinted benzyl mercaptan polymers were found to be complementary in size, i.e., the spatial effect, and chemically reactive towards benzyl mercaptan. Covalent rebinding with it and other various guest molecules were performed via re-forming disulfide bond at room temperature in protic solvents. The results proved that covalent recognition of templates are significantly more efficient than those based on other range of non-covalent interactions (Burri and Yu., 2016).

2.2 MATERIALS AND INSTRUMENTS

All commercially available solvents and reagents were used without further purification. Benzyl chloride, 1-propene-2-thiol, and azoisobutyronitril (AIBN) were purchased from Fluka analytical (Buchs, Switzerland). Ethylene glycol dimethacrylate (EGDMA), trifluoroacetic acid (TFA), diethyl ether, and 4-(tert-butyl) benzyl chloride were obtained from Sigma-Aldrich GmBH (Germany). Acetonitrile (ACN) and sodium chloride (NaCl) were obtained from Merck KGaA GmBH (Germany). 1,4-dithiothreitol (DTT), methanol (MeOH), benzyl mercaptan, sodium thiosulfate pentahydrate, and n-Hexane were purchased from Carl Roth GmBH (Germany), Fisher Scientific (U.K), Aldrich Chemie (Germany), J. T. Baker (Netherlands), and VWR Prolab international (France) respectively. Anhydrous sodium carbonate and sodium hydroxide pellets were purchased from AppliChem GmBH (Germany). For NMR analysis deuterated methanol (MeOH-D_4), deuterated chloroform ($CDCl_3$), and deuterated water (D_2O) were purchased from Cambridge Isotope Laboratories, Inc. (USA). As reported by us before (Burri and Yu., 2005) the proton spectra of the synthetic compounds were recorded on a Bruker DRX 600 spectrophotometer with 14.1 T field strength and 5mm xyz gradient TXI (H/C/N) probe was used. Topspin 1.3 and topspin 1.2 software were used to collect and analyze the data. High performance liquid chromatography (HPLC) was performed on Dionex chromatography interfaces model UCI-50, connected with the ASI-10U model auto sampler and UVD 170U model UV/VIS-detector. Chromolen 6.60 version software was used for control, process, and analysis on the spectra. A phenomenex, kromasil C-18 (5µm) column (150X4.6 mm) was eluted under 1mL/min flow rate. Milliq water used as a HPLC eluent was collected from the Barnsted nano pure diamond U.V^{TM} D11911 ultra-pure water system with Barnsted D3750 gamma irradiated hollow fiber filters. Ultraviolet lamp (model 3040, 400W power) from Photo chemical reactors Ltd (U.K) was used for the photo chemical radical polymerization (Burri and Yu., 2016; Burri and Yu., 2005).

All samples were employed on analytical HPLC system with two eluent systems: Eluent A is 0.1% TFA in acetonitrile and Eluent B is 0.1% TFA in milliq water. The elution rate was set for 1 mL/min over 20 min, starting conditions were eluent A (15%) and B (85%) and gradient reached at 18 min to eluent A (85%) and B (15%). From 18-20 min system went back to its initial state. The detection was carried out at 260nm UV channel (Burri and Yu., 2016).

2.3 EXPERIMENTAL SECTION

2.3.1 PREPARATION OF 3-BENZYLDISUFANYLPROP-1-ENE

2.3.1.1 Step 1: Preparation of prop-2-ene-1-sodiumthiolate solution (1)

As we reported before (Burri and Yu., 2016), in a 50-mL round bottomed flask equipped with a drooping funnel, a mechanical stirrer and a gas inlet, 2 g (50 mmol) sodium hydroxide in 5 mL water was charged. At room temperature 3.7g (50 mmol) of prop-2-ene-1-thiol was added under vigorous stirring. The viscous solution of prop-2-ene-1-sodiumthiolate (1) (shown in Figure 7) was obtained. In further, the solution was diluted with 1mL water and kept on ice bath (0^{o}C) (Burri and Yu., 2016).

2.3.1.2 Step 2: Preparation of Sodium benzyl thiosulfate (2)

As we reported before (Burri and Yu., 2016), in a 250 mL three necked round bottom flask equipped with a condenser, droping funnel and a mechanical stirrer, 6.3 g (50 mmol) benzyl chloride in 60 mL methanol were charged and refluxed for 2 h. Slowly water was added to this solution under stirring until turbidity developed. 15.5 g (62.5 mmol) sodium thiosulfate pentahydrate in 15 mL water was added over a period of 0.5 h. The resultant yellow colored solution was heated to reflux for an additional 4 h. The reaction mixture was cooled to room temperature. Methanol was removed with rotary evaporator, the remaining milky solution was diluted with water and extracted three times with *n*-hexane. The organic layers were discarded and evaporating the aqueous layer gave white powder. Subsequently the powders were dissolved in ethanol (2× 250 mL) and filtered off. Then solvent removal yielded 94% sodium benzyl thiosulfate (**2a,** in Figure 7) as white solid (Burri and Yu., 2016). The recorded NMR spectra shown in Appendix C. NMR: ^{1}H NMR (300 MHz, CD_3OD, δ): 7.36 (2H, Ar H), 7.26 (2H, Ar H), 7.19 (1H, Ar H), 4.39 (2H, CH_2).

The sodium *tert*-butyl benzylthiosulfate (III) was prepared with the same procedure by adding 5.09 g (20.52 mmol) of sodium thiosulfate pentahydrate to 3 g (16.42 mmol) (4-*tert*-butyl) benzyl chloride in 25 mL methanol, sodium 4-*tert*-butyl benzyl thiosulfate (**2b**) was yielded with 97% as a white solid powder (Burri and Yu., 2016). The recorded NMR spectra shown in Appendix C. NMR: ^{1}H NMR (300 MHz, CD_3OD, δ): 7.26 (4H, Ar H), 4.89 (2H, CH_2), 1.27 (9H, CH_3).

2.3.1.3 Step 3: Preparation of 3-benzyldisulfanylprop-1-ene (3)

As we reported before (Burri and Yu., 2016), a 250 ml round bottomed flask is charged with the 12 g (53 mmol) benzyl thiosulfate (**2a**) in 100 mL water cooled to 0ºC on ice bath. The cold prop-2-ene-1-sodiumthiolate solution (**1**) was added rapidly to reaction mixture with vigorous stirring for 10 min. The aqueous layer was extracted twice with 80 mL of diethyl ether. The organic layers were combined and dried briefly over granular anhydrous magnesium sulfate and filtered off. The removal of solvents gave 7.8 g (40 mmol, yield 75%) of 3-benzyldisulfanylprop-1-ene (**3**) as a crude oil. The recorded NMR spectra shown in Appendix C. NMR: ^{1}H NMR (300 MHz, DMSO-d_6, δ): 7.27 (1H, Ar H), 7.28 (2H, Ar H), 7.32 (2H, Ar H), 5.7 (1H, CH), 5.08 (2H, CH_2), 3.8 (2H, CH_2), 2.9 (2H, CH_2). (Burri and Yu., 2016).

2.3.2 POLYMER SYNTHESIS

As we reported before (Burri and Yu., 2016), the benzyl mercaptan imprinted polymers were prepared from radical polymerization reaction. Chemicals employed in this synthesis, amounts, reaction times, and polymerization methods are listed in the table 1. In a 15 mL glass tube, functional monomer (DBP) (IV) was dissolved in acetonitrile; crosslinking agent EGDMA and initiator 2,2-azobis(isobutyronitrile) (AIBN) were added to the mixture. The mixture was purged with a steam of nitrogen gas for 5 min. Glass tubes were sealed immediately. The MIPs from 1-8, 10A and 11A were irradiated under UV light at room temperature for 12 h and additionally 3 h at 80 ºC. MIP 10C and 11C were obtained by means of thermal polymerization at 80 ºC for 15 h. The resulting bulky polymers were grinded manually and dried in vacuum. The dried polymer particles were washed and extracted (Soxhlet extraction) with the 100 mL methanol. The supernatant was analyzed on HPLC and quantified for the unreacted part of the reaction mixtures. The control polymers i.e. non-imprinted polymers (NIP's 10C and 11C) were prepared with the same method under the thermal reactions, but prop-2-ene-1-thiol was used instead of 3-benzyldisulfanylprop-1-ene (Burri and Yu., 2016).

2.3.3 DISULFIDE REDUCTION

As we reported before (Burri and Yu., 2016), the disulfide bonds were reductively cleaved from the polymer particles by the following procedure as reported by (Takeuchi et al., 2006). Grinded polymer particles were suspended in methanol (100mL) with sodium borohydrate (20 mmol). The mixture was stirred for overnight at room temperature. The supernatant was analyzed on HPLC and quantified for the amount of template (benzyl mercaptan) that has been removed and given the possible binding sites in the MIP's (Burri and Yu., 2016).

2.3.4 POST MODIFICATION

As we reported before (Burri and Yu., 2016), the novel post modification on MIPs (and NIPs) was employed for the conversion of thiolic binding sites into thiolated binding sites. The certain amount of polymer particles (MIP/NIP) were suspended in water/MeOH solution and treated with the excess amount (1.5 fold excess to amount of polymer particles) of sodium hydroxide to convert the free thiols (-SH) into sodium thiolated form ($-S^-Na^+$). With this post-modifying process, it is only the chemical nature of binding sites but not any other physical properties of the MIPs/NIPs had been altered (Burri and Yu., 2016).

2.4 RESULTS AND DISCUSSION

2.4.1 TEMPLATE-FUNCTIONAL MONOMER ADDUCT

Benzyl mercaptan (BMT) is an organic sulfur compound. As shown in Figure 7, the 3-benzyldisulfanyl porp-1-ene (**3**) was synthesized by 3 step reactions. The 3-benzyldisulfanyl porp-1-ene (**3**) (BDP) was utilized as the functional monomer for preparing benzyl mercaptan imprinted polymers. The structure and purity of 3-benzyldisulfanyl porp-1-ene (**3**) was determined by means of NMR study. To increase the nucleophilic nature of the thiol group on the prop-2-ene-1-thiol, its thiol group had been converted in to the sodium thiolate form when being treated with sodium hydroxide (NaOH). The resulted reaction mixture contains 3-benzyldisulfanyl porp-1-ene and sodium sulfate (Na_2SO_3) as a by-product.

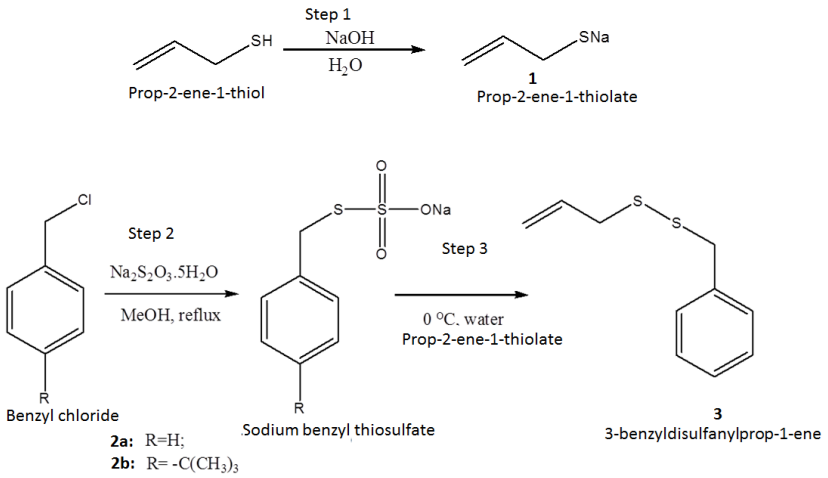


Figure 7: Synthetic route of 3-benzyldisufanylprop-1-ene (template-functional monomer adduct). . (Burri and Yu., 2016).

The oil product was separated from the aqueous phase and extracted with diethyl ether. Evaporation of solvents led to pure colorless oil product in high yield (95%). Their NMR spectra confirmed the correct chemical structure of the designed covalent bonded template-monomer moiety with 1:1 molar ratio, i.e., the functional monomer (3-benzyldisulfanyl porp-1-ene), for covalent imprinting, which improved the homogeneity of MIPs' binding sites as well.

2.4.2 OPTIMIZATION OF CO-POLYMERIZATION

Several factors such as amount of crosslinking agent, type of polymerization (photo or thermal initiation), reaction time, temperature, solvents and co-solvents had great influences on the co-polymerization process. Imprinting with disulfides hasn't been evaluated yet, only few research work had been reported using disulfides in molecular imprinting, however followed by recognition of the templates via non-covalent interactions. In this process, polymerization conditions, molar ratios of reaction mixtures, the effect of crosslinker, and reaction time were optimized.

In this project we have prepared two types of polymers, one with the template mediated polymers called molecularly imprinted polymers (MIPs) and other polymers prepared without using the templates called as non-imprinted polymers (NIPs). EGDMA used as a crosslinker for co-polymerizing/crosslinking the BDP. The molar ratio of BDP and EGDMA plays an important role in the polymerization, template removal, tuning the binding sites, rebinding studies, and rebinding selectivity. In the previous section 1.5.1.2, influence of crosslinkers on imprinting have been discussed. Hence, MIPs and NIPs have been synthesized with different molar ratios at different temperatures, as presented in Table 1.

It is important to quantify the released BDP, since template molecules may cleave from the growing polymeric network during polymerization. MIPs particles were extracted with the methanol. During this processes unreacted BDP, EGDMA, and oligomers were extracted. The extracts were analyzed on HPLC and then quantified by using standard curve of BDP.

Table 1: Polymerization recipe and conditions of MIPs and NIPs. The BDP used in all MIPs synthesis and prop-2-ene-1-thol (PET) used in NIPs.

Polymer	**BDP (mmol)**	**EGDMA (mmol)**	**AIBN (mmol)**	**Solvent (mL)**	**Reaction conditions**	**Time (h)**
MIP1	2	10	0.183	3	UV lamp	2
MIP2	2	10	0.183	3	UV lamp	4
MIP3	2	20	0.183	3	UV lamp	2
MIP4	2	20	0.183	3	UV lamp	4
MIP5	2	30	0.183	3	UV lamp	5
MIP6	2	40	0.183	3	UV lamp	5
MIP7	2	30	0.183	3	UV lamp	6
MIP8	2	40	0.183	3	UV lamp	6
MIP9A	3	15	0.183	3	UV lamp	12
MIP10A	3	30	0.183	3	UV lamp	12 (+3)
MIP11A	3	45	0.183	3	UV lamp	12 (+3)
MIP10C	3	30	0.183	3	Thermal at 80^{o}C	12 (+3)
MIP11C	3	45	0.183	3	Thermal at 80^{o}C	12 (+3)
NIP10C	3(PET)	30	0.183	3	Thermal at 80^{o}C	12 (+3)
NIP11C	3(PET)	45	0.183	3	Thermal at 80^{o}C	12 (+3)

2.4.3 TEMPLATE REMOVAL

The imprinted template (BMT) covalently linked through the disulfide bonds to the polymeric network. In order to remove the BMT, disulfide bonds are to be cleaved. Typically disulfide bond reduction can be performed by reducing agents such as DTT, $NaBH_4$, and $LiAlH_4$. In this study disulfide bonds were straightforwardly reduced from the polymer complexes using with $NaBH_4$-methanol solution (Mukawa et al., 2003), which resulted in thiol containing binding sites (BS-SH). These binding sites are complementary towards BMT or structurally BMT related compounds (Takeuchi et. a.l., 2006). To quantify the BS-SH, polymer particles were suspended in $NaBH_4$-methanolic (20 mmol of sodium borohydrate in 100 mL methanol) solution and stirred for 24h at room temperature, subsequently the supernatant was analyzed on HPLC and quantified for BMT with the point analysis method. Standard curve of BMT was prepared on HPLC from the known/designed concentration of commercially available BMT. Figure 8 illustrates the overview of the amounts of BDP used in imprinting and the created binding sites in the polymeric network. In table 2, the amounts of reduced BMT, particles weight, and binding sites are listed.

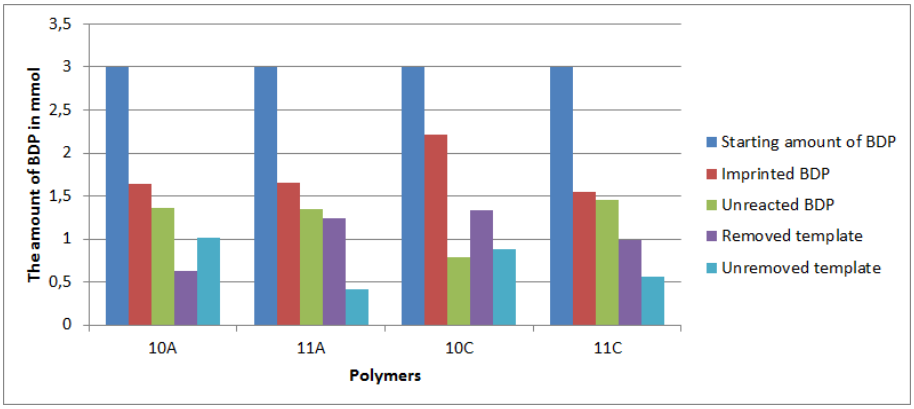


Figure 8: Overview of the amounts of BDP which are the initial, imprinted, unreacted, removed, and non-removed amount during and after the imprinting process (Burri and Yu., 2016).

The binding sites (BS-SH) are widely distributed and are quantified as available maximum binding sites (B_{max}) of 157 µmol g^{-1}, 202 µmol g^{-1}, 359 µmol g^{-1}, and 141 µmol g^{-1} in MIP10A, 11A, 10C, and 11C respectively. While increasing amounts of crosslinking agent increases the crosslinking density of the network polymers which results in less available binding sites in MIP11A and 11C. Thermally polymerized polymers yielded almost two fold excess binding sites than the photochemical initiated polymers.

Table 2:The amount of templates (benzyl mercaptan) removed from the imprinted polymers, weight of polymer particles, and available binding sites in MIP10-11 A & C.

Polymer	**Amount of reduced template (mmol)**	**Particles Wt. (g)**	**Binding sites (B_{max}) (µmol g^{-1})**
MIP10A	0.62	3.94	157
MIP11A	1.24	6.14	202
MIP10C	1.33	3.70	359
MIP11C	0.99	6.98	141

2.4.4 REBINDING AND SELECTIVE STUDIES

The recognition properties of MIPs and control polymers (NIPs) were carried out both covalently and non-covalently in protic solvents. The rebinding and selective studies were performed by using benzyl mercaptan, phenol, sodiumbenzyl thiosulfate, and sodium *tert*-butyl-benzyl thiosulfates in methanol. The chemical structures of four templates are shown in Figure 9**.** The templates BMT and phenol recognition were accomplished non-covalently with benzyl mercaptan and phenol and covalently with sodium benzyl thiosulfate and sodium *tert*-butyl-benzyl thiosulfates by forming disulfide bridges through the reactive/functional sites in the MIPs.

Benzyl mercaptan had been expected to rebind the MIPs via oxidations of thiol groups between BMT and binding sites. However, we have proven that oxidation takes place in between the two BMT molecules and forming benzyl disulfides. Hence binding strategy of BMT was in fact confirmed through non-covalent binding, i.e., hydrogen bond forming ability of thiol group in BMT interacts with the free -SH functional sites of MIPs. In table 3 the binding capacities of BMT and phenol towards the MIPs and NIPs are shown via the ratio in percentage between the binded or bonded amount [B] to their maximum amount of the analytes that can be binded, [B_{max}], whereas [B] is the difference between initial- and free-amount of the anaylytes [I] and [F]. The results suggest that BMT covalently imprinted MIPs possess high binding strength towards BMT, i.e., MIP10A (36%) and MIP10C (37%) and low binding strengths in MIP11A (14%) and MIP11C (2%), which can be attributed to the degree of crosslinking: MIP10A and MIP10C had been obtained with 50% higher amount of EGDMA than MIP11A and MIP11C. By adding excess amount of crosslinkers significantly decreases binding capacities of BMT, however phenol showing the similar binding strengths, for these MIPs (10C, 11A, and 11C) 12%, 11.8%, and 12.6% were pronounced respectively (Burri and Yu., 2016).

Table 3:Binding strengths (shown in percentages: [B]/[Bmax]%, (B=I-F) of various templates with MIPs and NIPs, [B], the binded or bonded amount of analytes; [I], the initial amount of the analytes (Burri and Yu., 2016).

Polymers	**Sodium benzyl thiosulfate** **[B]/[B_{max}]%**	**Sodium tertiary butyl benzyl thiosulfate** **[B]/[B_{max}]%**	**Benzyl mercaptan** **[B]/[B_{max}]%**	**Phenol** **[B]/[B_{max}] %**
MIP10A	67.5	48.6	36.8	-
MIP10C	39.1	23.9	37.4	11.1
MIP11A	71.3	33.9	13.9	11.9
MIP11C	47.8	33.8	2.1	12.8
NIP10C	22.1	6.9	27.0	20.1
NIP11C	27.2	14.7	29.8	18.4

This indicates that spatial effects play a more important role here than binding strengths, since in principle hydroxyl groups in phenol should have stronger hydrogen bonding ability than those in BMT, while here comparable binding strengths are found. For MIP10A and MIP10C, the almost same binding strength on BMT suggests that similarly densed network were obtained on either thermal conditions or UV light driven polymerization, when having relatively low amount of crosslinker; while when having increased amount of 50% than this, MIP11A and MIP11C discriminate BMT dramatically (14% versus 2%), proving that thermally crosslinking results in much higher degree of crosslinking than UV-radiation does. However MIP11A and MIP11C binds phenol at the same level, this might be due to that phenol possesses smaller molecular size that BMT does, therefore the limited space for BMT turned out to be unlimited to phenol yet. Especially when comparing the rebinding capacity among MIPs and NIPs, we can see that guests in smaller molecular sizes are binded independently on the amount of crosslinkers when they bind either BMT or phenol. These had also proven that MIPs and NIPs recognize the templates with non-covalent interactions with low binding strengths.

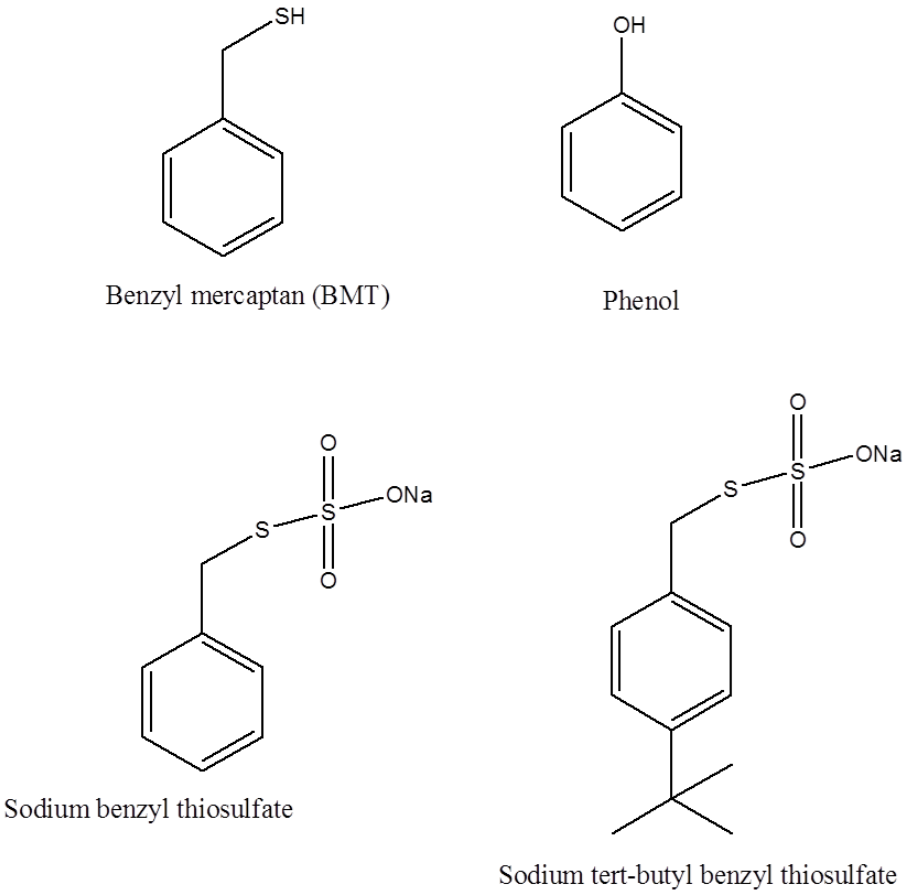


Figure 9: Template structures of benzyl mercaptan, phenol, sodium benzyl thiosulfate and sodium tert-butyl benzyl thiosulfate.

As we reported us before (Burri and Yu., 2016); a novel method has been introduced for binding sites modification in MIPs and NIPs without losing shape, structure, and molecular memory of MIPs. The nature of thiols acts as weak nucleophiles which attacks disulfides and involves in thiol-disulfide exchange strategy. Although these reaction is common in biological context (denaturation of proteins), it cannot sustain in small organic molecules for *in vitro* experiments. Therefore; the thiol-groups (-SH) in binding sites were converted in to thiolate ($-S^-Na^+$) form to facilitate the covalent recognition of templates (sodium benzyl thiosulfate and sodium *tert*-butyl benzyl thiosulfate) by reforming disulfide bonds. Control polymers (NIPs) were tested to determine the role of template structures in selective binding (Burri and Yu., 2016).

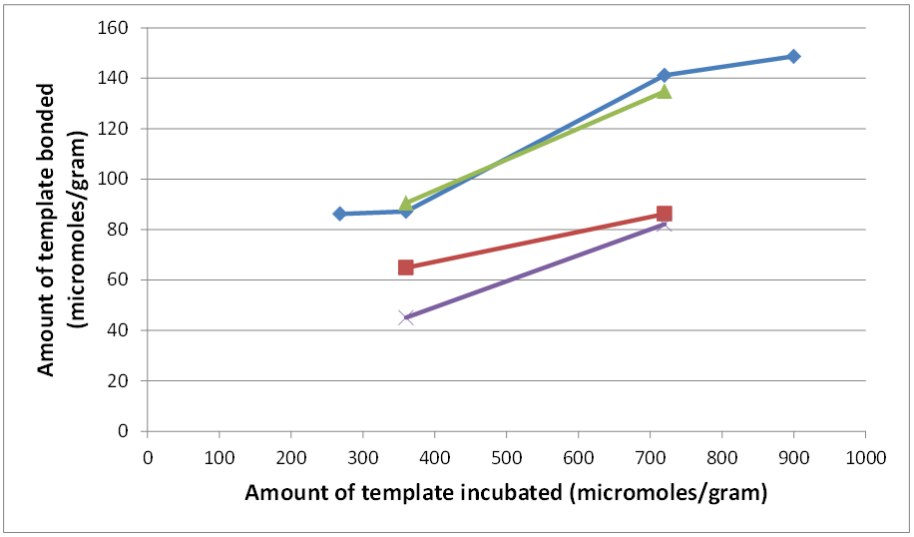


Figure 10: Binding strengths of sodium benzyl thiosulfate ( ), sodium tertiary butyl benzyl thiosulfate ( ), phenol ( ) and benzyl mercaptan ( ) in MIP 10C, as presented in our published article (Burri and Yu., 2016).

To evaluate post modification effects on MIPs and NIPs towards rebinding, at room temperature post-modified imprinted polymer particles (PMIPs) were incubated with sodium benzyl thiosulfate and sodium *tert*-butyl benzyl thiosulfate templates in 0.7, 1, 2, and 2.5-fold excess to binding sites (Burri and Yu., 2016.

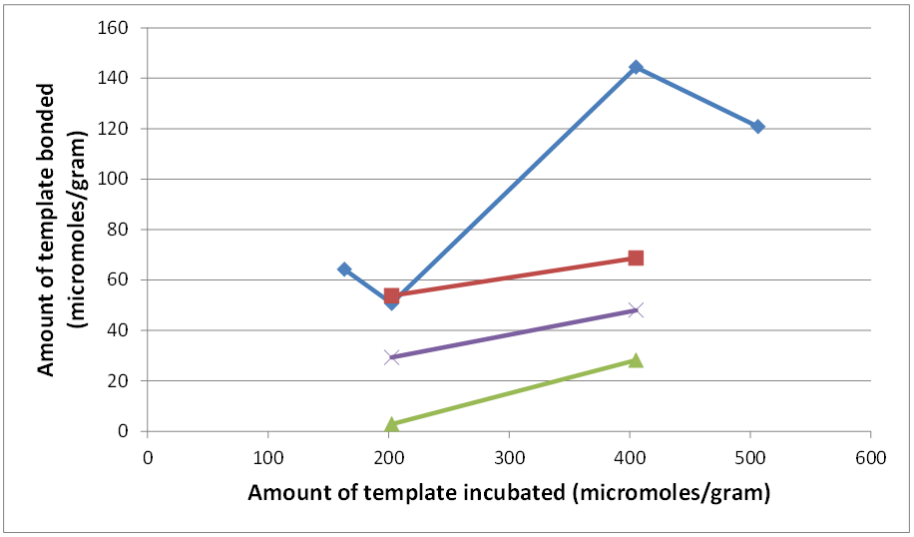


Figure 11: Binding strengths of sodium benzyl thiosulfate ( ), sodium tertiary butyl benzyl thiosulfate ( ), phenol ( ) and benzyl mercaptan ( ) in MIP 11A. (Burri and Yu., 2016).

Binding strengths were calculated. We observed that PMIPs provide good recognition and binding capacities to benzyl mercaptan moieties in sodium benzyl thiosulfate (see Figure 10 -13) (Burri and Yu., 2016). PMIPs are found bonding BM moiety in benzyl thiosulfates more significantly than the NIPs does, proving that sodium benzyl thiosulfate had been successfully covalently re-bonded with higher strengths on modified particles through disulfide linkage. This was found to be 1.8, 5, and 20 fold excess than BMT which was directly recognized non-covalently in MIP10A, 11A and 11C respectively.

Sodium *tert*-benzyl thiosulfate salt showing significantly less bonding even though it's having same functional group and being able to react with the active sites through same strategy; these results imply the imprinting effect. Nevertheless, BMT and phenol templates also showing comparatively less binding affinity with MIPs through non-covalent interactions (hydrogen bonding), indicating that recognition of templates with covalent bonds (disulfides) are more effective than non-covalent bonds. Such study supports that covalent recognition is fast, efficient, and concentration independent. More than 60% of sodium benzyl thiosulfate bounded at 2-fold excess of template incubated than maximum binding sites. Figure 10, Figure 11, and Figure 12 present the binding strengths of templates in MIP10C, MIP11A and MIP11C respectively.

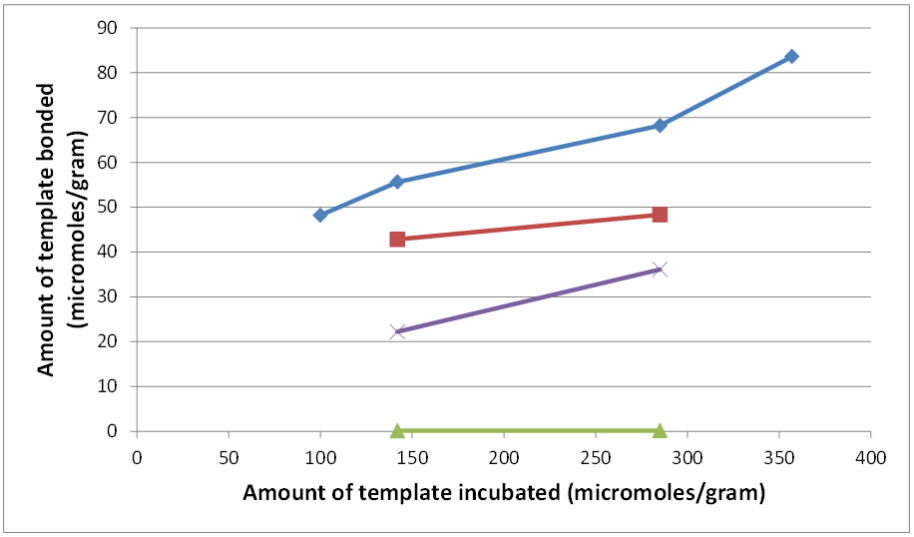


Figure 12: Binding strengths of sodium benzyl thiosulfate ( ), sodium tertiary butyl benzyl thiosulfate ( ), phenol ( ) and benzyl mercaptan ( ) in MIP11C. (Burri and Yu., 2016).

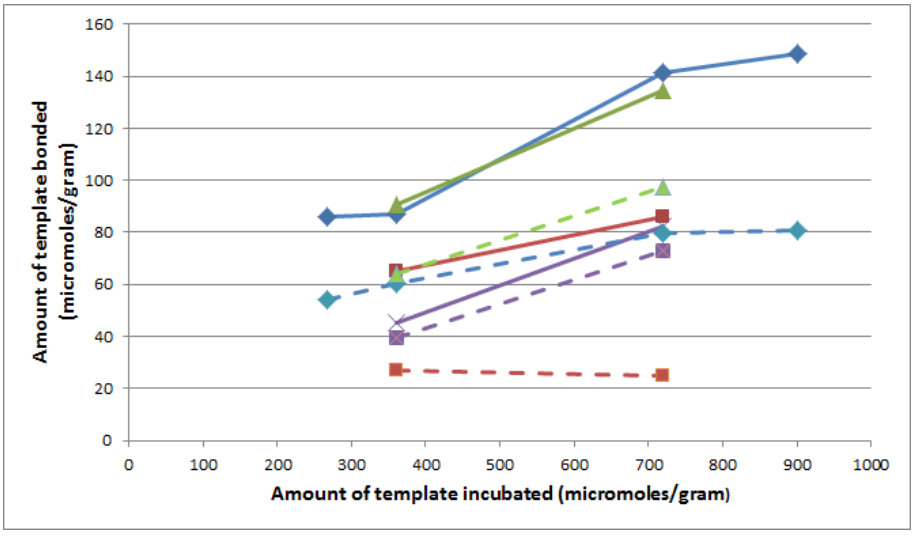


Figure 13: Relative binding strengths of sodium benzyl thiosulfate ( ), sodium tertiary butyl benzyl thiosulfate ( ), phenol ( ) and benzyl mercaptan ( ) in MIP10C (straight lines) and NIP10C (dashed lines), (Burri and Yu., 2016).

As described above the binding affinity towards MIPs, concurrently recognition and binding affinities of four templates have been tested with control polymers (NIPs). The NIP particles binding sites were also modified under the same way which was performed on MIPs. Figure 13 shows relative binding affinities of template molecules in MIP10C and NIP10C; while Figure 14 reveals the relative binding affinities of template molecules in MIP11C and NIP11C. The binding affinities of templates sodium benzyl thiosulfate and sodium *tert*-benzyl thiosulfate serve the covalent recognition. BMT and phenol serve non-covalent recognition showing high affinities towards the MIPs comparatively.

NIPs, however shows lower affinities compared with the covalent recognition. These results supporting template specific cavities in the imprinted polymers (MIPs) are recognizing the same templates or structurally related templates; whereas NIPs are lacking those template specific cavities when even having the same functional groups as the MIPs to form the covalent and non-covalent interactions.

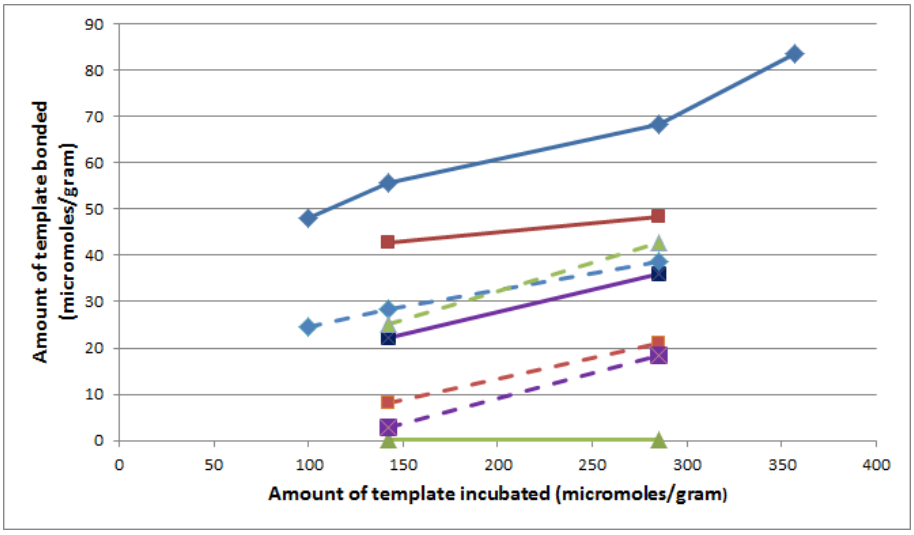


Figure 14: Relative binding strengths of sodium benzyl thiosulfate ( ), sodium tertiary butyl benzyl thiosulfate ( ), phenol ( ) and benzyl mercaptan ( ) in MIP11C (straight lines) and NIP11C (dashed lines) (Burri and Yu., 2016).

2.5 CONCLUSIONS AND PERSPECTIVES

We employed a novel method for creating synthetic receptors with molecular imprinting technique via covalent interactions (disulfide bonds) and successfully modified binding sites through post modification process to facilitate recognition on the templates and structurally related templates with covalent bonding by reforming disulfide bonds. The results prove that post modification method makes recognitions effective and significant without loss of molecular memory in binding sites. These kinds of tunable binding sites are more advantageous in selective choice of templates. The results show that covalent recognition with disulfides are fast, efficient, and concentration independent when recognition was carried out in diluted solutions. The covalent imprinting with disulfides and recognition can be carried out in all atmospheric conditions and both aprotic and protic solvents, and even in water in principle as long as the analytes are water-soluble. The various templates of organic molecules and proteins containing sulfur atom are able to form disulfide bonds and therefore get recognized. And additionally recognition in protic solvents will be used to develop novel method for creating protein based artificial receptors and recognize them in biological systems, also possibly in aqueous systems.

CHAPTER 3. RECEPTOR-LIKE FMOC-PROTECTED CYSTEINE IN ORGANIC POLYMERS

3.1 MOTIVATION

As described in Chapter-2; post modification on covalently imprinted benzyl mercaptan synthetic polymers can efficiently recognize the templates by forming reversible disulfide bonds with high binding strengths. So, we believed that these disulfides based covalent imprinting and recognition templates by reforming such same bonds are highly attractive. Based on such investigations, we anticipated preparing protein based imprinting structures with covalent bonds. The amino acids are building blocks for proteins and play an important role in all biological process. In general, most of the peptides contain cysteine amino acid in their sequences and are able to form disulfide bridges. These disulfide bridges play an important role in protein folding further these protein folding is very unique to functioning the protein. Therefore, we have considered preparing cysteine based imprinted polymers with disulfide bonds to improve applications in molecular imprinting of proteins. We have also considered that recognition of cysteine in water will increase attention for developing amino acids/protein sensors *in vivo* or in biological media.

As we have introduced in our published work (Burri and Yu., 2015); A novel synthetic N-(9-fluorenyl methoxy carbonyl) - L-Cysteine (Fmoc-Cys-OH) receptor was prepared by co-polymerizing with (9-fluorenyl methoxy carbonyl)-S-(1-propene-2-thiol)-L-Cysteine (Fmoc-Cys(SCH_2CHCH_2)-OH) under ultraviolet radiation for 15 h at room temperature and further keeping heated at 55 ^oC for 3 h. Control polymers (non-imprinted polymers) were prepared under the same way however, without having template. Consequently, disulfides in the polymers were reductively cleaved with lithium aluminum hydride ($LiAlH_4$) from the imprinted polymers. The imprinted polymers selectively recognized Fmoc-Cys-OH with high binding constants in aqueous and protic solvents by thiol-disulfide exchange reactions (Burri and Yu., 2015). Further to confirm the reproducibility of imprinted polymers and covalent rebinding, particles were extracted with methanol and reduced to disulfides to analyze the covalently rebind. Furthermore; repeated rebinding studies with imprinted polymer particles showed constant binding strengths and recognition properties. Figure 15 discloses the schematic view of molecular imprinting procedure of modified cysteine amino acid.

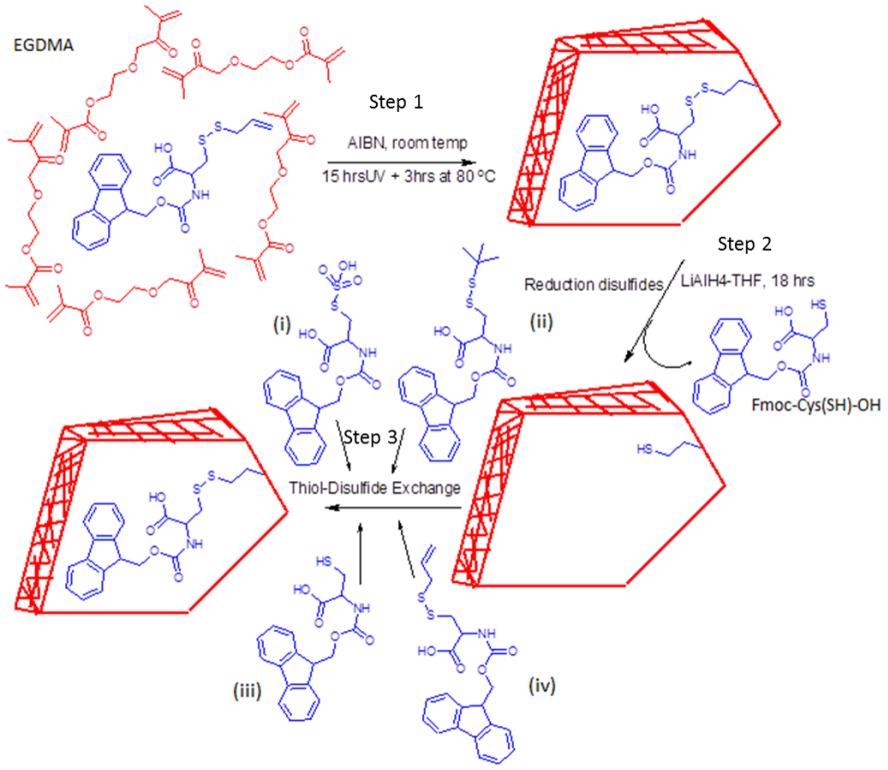


Figure 15: Schematic representation of molecular imprinting of modified cysteine. Step1: polymerization, step 2: removal of templates, and step 3: recognition of templates. Templates used for recognition: (i) Fmoc-Cys(SO3H)-OH, (ii) Fmoc-Cys(S-t-Bu)-OH, (iii) Fmoc-Cys-OH, and iv) Fmoc-Cys(SCH_2CHCH_2)-OH. (Burri and Yu., 2015).

3.2 MATERIALS AND INSTRUMENTS

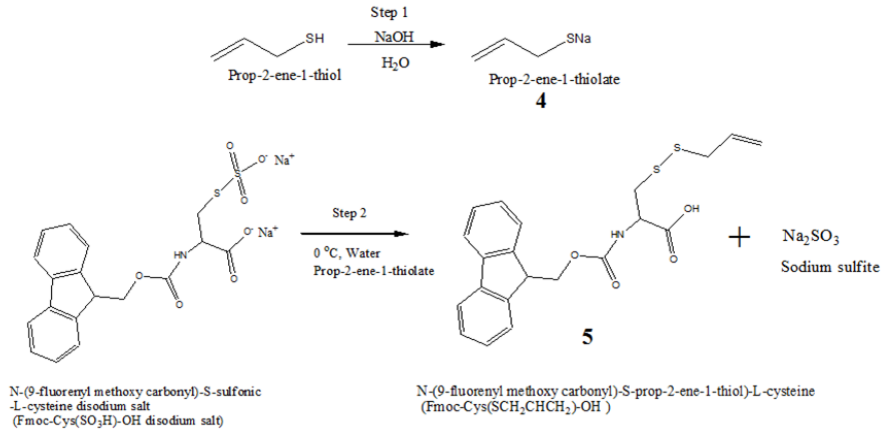
For the same solvents/reagents and analytical instruments (HPLC/NMR) as used/introduced in the session of Materials and Instruments in Chapter- 2, they are not furthermore described here. Additionally, tetrahydrofuran (THF), trifluroacetic acid (TFA), piperidine and N,N-diisopropylethylamine (DIPEA) were obtained from Sigma-Aldrich GmBH (Germany). 2M Lithium aluminum hydride ($LiAlH_4$) solution in THF and trisisopropylsilane (TIS) were purchased from Aldrich Chemie (Germany). (Benzotriazol-1-yl)-N,N,N´,N´-tetramethyluronium (HBTU) and N-hydroxybenzotriazole monohydrate (HBTU) were obtained from Advanced Chemtech (USA). Amino acids N-(9-fluorenyl methoxy carbonyl)-S-tert-butylthio-L-cysteine (Fmoc-Cys(S-*t*-Bu)-OH) and N-(9-fluorenyl methoxy carbonyl)-S-sulfonic–L-cysteine disodium salt (Fmoc-Cys(SO_3H)-OH) were ordered from Bachem AG (Switzerland). The samples were employed on HPLC system with two eluent systems: Eluent A (0.1% TFA in acetonitrile and Eluent B (0.1% TFA in milliq water). The

elution rate maintained 1 mL/min over 35 min, starting conditions were eluent A (15%) and B (85%) and gradient reaching at 18 min to eluent A (85%) and B (15%). From 18-20 min system reached to its initial state. The detection was carried out at 280nm UV channel (Burri and Yu., 2015).

3.3 EXPERIMENTAL SECTION

3.3.1 PREPARATION OF FMOC-CYS(SCH_2CHCH_2)-OH (5)

As we previously described (Burri and Yu., 2015); 2.5 mmol of prop-2-ene-1-thiol (185 mg) were added dropwise to 2.5 mmol (100 mg) of sodium hydroxide in 3 mL of water in a 25 mL round bottomed flask. The viscous solution of prop-2-ene-1-sodiumthiolate **(4)** was obtained upon 3 h with vigorous stirring at room temperature. Reaction mixture was diluted with water (3 mL) and kept on ice bath. A 250 mL three necked round bottomed flask was charged with 2.5 mmol of Fmoc-Cys(SSO_3H)-OH sodium salt (1.17 g) in 50 mL water. The prepared prop-2-ene-1-sodium thiolate solution **(4)** was added rapidly to reaction flask with vigorous stirring at 0 °C. Reaction was progressed for 30 min (Burri and Yu., 2015). Reaction scheme is shown in Scheme 1.



Scheme 1: Synthetic route of functional monomer (Fmoc-Cys(SCH_2CHCH_2)-OH).

A white thick precipitation was formed in the reaction mixture. Further reaction mixture was washed with dilute HCl and extracted with ethyl ether (3×150 mL). The organic layers were combined and dried under anhydrous $CaCO_3$ and filtered off. The filtrate was evaporated, 2.15 mmol of Fmoc-Cys(SCH_2CHCH_2)-OH **(5)** was yielded as a color less oil. The recorded NMR spectra's presented in Appendix C. ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 7.8 (2H, Ph-H, Fmoc); 7.7 (2H, Ph-H, Fmoc); 7.4 (2H, Ph-H, Fmoc); 7.3 (2H, Ph-H, Fmoc); 5.7 (1 H, ethylene); 5.2 (2H, ethylene); 4.3

(4 H, CH_2); 4.02 (1H, CH); 3.3 (2H, CH_2); 3.1 (1H, CH^{α} Cys); 2.9 (1H, CH^{α} Cys). ^{13}C NMR (100 HZ, DMSO-d_6) δ (ppm): 156.4 (C=O); 144.2 (Ph); 141.1 (Ph); 133.9 (1-ethylene); 128 (Ph); 125.5 (Ph); 119.1 (1-ethylene); 66.1 (CH_2), 55.3 (C^{α}); 47 (CH); 41.2 (CH_2); 40.3 (CH_2) (Burri and Yu., 2015).

3.3.2 PREPARATION OF FMOC- PROTECTED DIPEPTIDE (FMOC-PHE-CYS(S-T-BU)-$CONH_2$) (6)

Fmoc-protected dipeptide (Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$) was synthesized manually on solid phase Fmoc-strategy. Synthesis of dipeptide was carried out in 4 steps which includes resin-swelling, deprotection, amino acid activation, and coupling and cleavage of final peptide from resins. 1 g of rinkamide resin (loading capacity 0.65 mmol/g) was taken into a 50 mL peptide synthesis reactor, then 15 mL of DMF was added and shaked thoroughly over 30 min. 10 mL of 25 % piperadine in DMF was added to swollen resin and mixed thoroughly for 20 min to deprotect Fmoc-group from the resin, deprotection was monitored with Kaiser Test. Subsequent amino acid coupling was carried out with 4 equivalent to resin loading capacity of amino acid, 2.6 mmol (1.5 g) of Fmoc-Cys(S-*t*-Bu)-$CONH_2$ was dissolved in 10 mL DMF, 0.5 M HBTU/HOAt and 1 M DIPEA were added to activate the carboxylic group. After 5 min activated mixture was added to resin and mixed on shaker upon 2 h. The coupling reaction was monitored with Kaiser Reagent. The second amino acid, 4 equivalent, 2.6 mmoles (0.58 g) of Fmoc-Phe-OH coupling was carried out under the same way except final deprotection step. Resin was washed with DMF after every deprotection and coupling step. However final washing step was carried out with DCM. Peptide cleavage was performed with TFA-TIS-H20 (95:2.5:2.5) mixture. 10 mL of cleavage mixture was added to resin and mixed upon 2.5 h and consequently the resin was filtered off and the produced peptide was precipitated in cold diethyl ether. Lyophilization of them resulted in dipeptide (Fmoc-Phe-Cys[S-*t*-Bu]-$CONH_2$) as white powder (Burri and Yu., 2015).

3.3.3 PREPARATION OF POLYMERS

As we noted in our published article (Burri and Yu., 2015); Polymers were synthesized by free radical polymerization process as illustrated in step one in Figure 15. 1.3 mmol of Fmoc-Cys(SCH_2CHCH_2)-OH (0.545 g) was dissolved in 26 mmol of DMF (2 mL) in a 50 mL glass tube. 26 mmol of EGDMA (5.2 mL), 0.4 mmol of AIBN (65 mg), and 260 mmol of THF (21 mL) were added to it. The reaction tubes were sonicated shortly and degassed with N_2 gas for 5 min. The reaction tubes are sealed and irradiated under UV light for 15 h at room temperature. Afterwards the tubes were incubated for 3 h at 80^{o}C. The resulted bulk polymer materials were finely grinded and dried at 40^{o}C for overnight. Polymer particles were extracted with methanol (100 mL) in soxhlet extraction over 12 h. The extractives are examined on HPLC. The control polymers (NIPs) were prepared with the same method without

adding template molecules, however prop-2-ene-1-thiol was added for introducing thiol groups in NIPs (Burri and Yu., 2015). The contents of the chemicals employed in imprinting process are given in Table 4. The prepared imprinted polymers denoted as CMIP and non-imprinted (control) polymers denoted with CNIP (C stands for cysteine).

3.3.4 TEMPLATE REMOVAL

Disulfide bonds in the polymeric particles were reductively cleaved with reducing agents. In a round bottomed flask, polymer particles were suspended in 2M $LiAlH_4$ in THF solution. The reaction mixture was stirred for 18 h at room temperature, further diluted with sulfuric acid, and then methanol was added dropwise to reaction mixture. Polymer particles were filtered off and supernatant was analyzed on HPLC (Burri and Yu., 2015).

3.3.5 BINDING AND SELECTIVITY STUDIES

Batch rebinding and selectivity experiments were carried out in methanol, however water was used for Fmoc-Cys(SO_3H)-OH. In a series of 50 mL centrifuge tubes containing CMIPs (0.650 g) or CNIPs (0.625 g), the mixtures were incubated with known amount (10, 20, 30, and 40 µmole) of various templates includes Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-*t*-Bu)-OH, Fmoc-Leu-OH, and Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$. The reaction tubes were placed on shaking table for 18 h, and then centrifuged. The supernatant was analyzed on HPLC (Burri and Yu., 2015).

3.4 RESULTS AND DISCUSSION

3.4.1 PRINTED MOLECULE

Cysteine is an essential amino acid; and contains carboxylic, amino, and thiol group. The thiolic side chain of cysteine acts as nucleophile and participates in many of enzymatic process. Oxidation of thiolic groups on two different cysteine molecules forms derivatives of cysteine. These disulfide bridges play an important role in protein folding and functioning. We have considered various amino acids for imprinting covalently, however cysteine is attractive to facilitate forming disulfides in recognition process. Therefore, we designed a functional monomer Fmoc-Cys (SCH_2CHCH_2)-OH, which is protected by Fmoc- group and connected to prop-2-ene-1-thiol through disulfide bridge, as presented in Figure 16.

In general, template and monomer complex plays a crucial role in imprinting process and more stable complexes are highly attractive to obtain highly selective MIPs (Karim et al., 2005). Such a template (Fmoc-Cys-OH) covalently bonded functional

monomer (FM) take great advantages of disulfide linkage, thus precious stoichiometry exists. Consequently, this product is highly stable and further experiments will be conducted at high temperatures. The designed functional monomer can be applied in diverse applications as polymerization are based on the vinyl functional group, and such functional group contains Fmoc-protection that was used in incorporating peptide growing chain on solid phase peptide synthesis in form of disulfide linkage useful in template removal and recognition covalently. The synthetic route of this compound is straight forward and described in experimental section. Purity and structural determination of the synthesized product was characterized on NMR and HPLC.

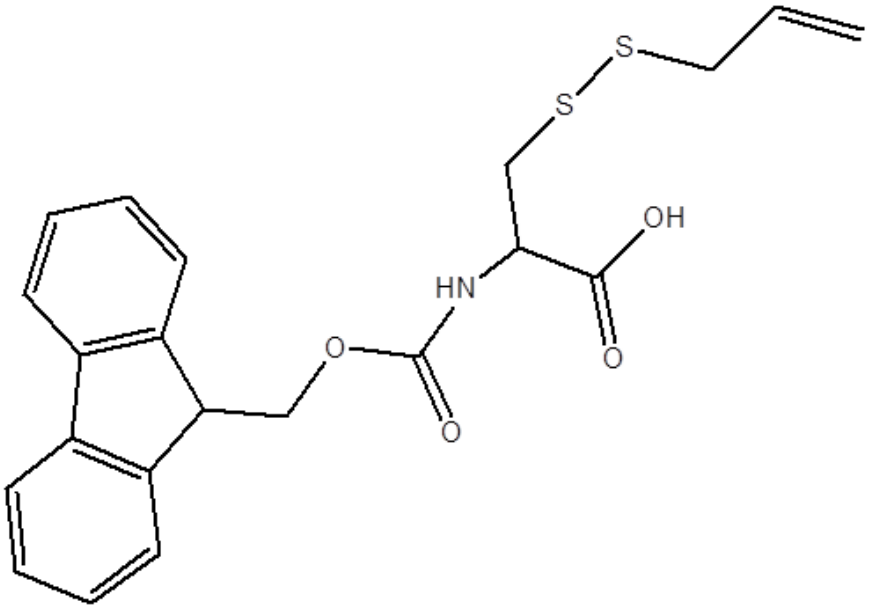


Figure 16: Chemical structure of the functional monomer [N-(9-fluorenyl methoxy carbonyl)-S-prop-2-ene-1-thiol)-L-cysteine]. Fmoc-protected cysteine with vinyl functionality connected with a disulfide bridge.

3.4.2 OPTIMIZATION IN DEGREE OF CROSSLINKING

Crosslinkers are the main parts of the back bones of the polymeric network and occupies around 80-95 mol % of total amount. So the nature of the crosslinkers and degree of crosslinking plays a key role in non-specific interactions taking place with binding analytes during recognition, and maintains template specific cavities in binding sites of imprinted polymers. The polymers were prepared by using different amounts of crosslinkers, solvents at different temperature and with different reaction time. The molar ratios of all components are given in table 4. Our previous results,

which were shown in section 2.4.2 in optimization of crosslinking density for benzyl mercaptan, found the optimal molar ratios between functional monomer and crosslinkers and the reaction time. For CMIP1, CMIP2, and CMIP3 shorter reaction time (6 h) and molar ratios of FM: EGDMA of 1:20, 1:40, and 1:60 were employed respectively.

Table 4: The contents of the chemicals employed in the imprinting process. The control polymers also prepared from CNIP1 to CNIP9 with the same molar ratios as those of CMIP1-CMIP9.

Polymers	**(FM) (mmol)**	**EGDMA (mmol)**	**DMF (mmol)**	**THF (mmol)**	**Time (h)**	**Imprinted FM (mmol)**	**Unreacted FM (mmol)**
CMIP1	0.5	10	39	---	6	0.21	0.29
CMIP2	0.5	20	39	---	6	0.26	0.24
CMIP3	0.5	30	39	---	6	0.30	0.20
CMIP4	0.8	4	39	---	12	0.28	0.52
CMIP5	0.8	8	39	---	12	0.28	0.52
CMIP6	1.5	15	26	260	15+(1)	0.60	0.90
CMIP7	1.2	12	26	260	15+(2)	0.50	0.70
CMIP8	1.3	13	26	260	15+(3)	0.45	0.85
CMIP9	1.3	26	26	260	15+(3)	0.52	0.78
CNIP8	1.3	13	26	260	15+(3)	-NA-	-NA-
CNIP9	1.3	26	26	260	15+(3)	-NA-	-NA-

In order to estimate amount of FM utilized in imprinting process, polymer particles were extracted with methanol. It is confirmed that around 40-60% of FM was utilized in imprinting procedure and remaining amount was washed out after polymerization. Subsequently, reaction time was prolonged from 6 h to 12 h, under the FM and crosslinker ratio of 1:5 (CMIP4) and 1:10 (CMIP5).

The largest amount of crosslinker was added in CMIP3 and may have yielded highest crosslinking density, so template removal became hard due to that the removed templates were trapped in densed polymeric network. Therefore, porogenic solvent was employed in later imprinting process. The THF was used as porogen to make porous materials to migrate solute particles. Other polymers (CMIP6-9) were prepared using 10 times excess porogen than solvent, while other parameters were changed accordingly to obtain optimal conditions for imprinting process. In synthesis of CMIP6-8, ratios between FM and crosslinker were kept the same, i.e., 1:10, while the reaction time varies. After 15 h polymerization reaction, CMIPs were further incubated at 80 ^{o}C for 1 h (CMIP6), 2 h (CMIP7) and 3 h (CMIP8). CMIP9 was prepared under the same conditions as used for CMIP8, however FM and crosslinker ratio was corrected to 1:20. The control polymers (CNIP8 and CNIP9) were prepared via the same as above mentioned, however FM molecule Fmoc-Cys(SCH_2CHCH_2)-OH was replaced by prop-2ene-1-thiol.

3.4.3 DISULFIDE REDUCTION

The template molecules in CMIPs were connected with polymeric networks through a disulfide bridge. Reductively cleaving this disulfide bond leaves specific cavities (binding sites) for the guest molecules (Fmoc-Cys-OH, and alike) in the CMIPs. As previously used, reducing agent $NaBH_4$ in methanol solution was applied to reduce disulfide bonds in CMIP1-CMIP9, and the supernatant was analyzed on HPLC for determining the free concentration of the guests, [F]. However, the reduced template was not found in supernatant, this could happen due to that the reduced templates might have stick in the polymeric networks or they contain not enough pores on polymer surfaces else stronger reducing agents are needed. On the other hand, CMIP6-7 were prepared by using co-solvents THF (used as a porogen). So strong reducing agents $LiAlH_4$ were used in CMIP1-9, thus the removed templates were found in supernatants and further amount of template was characterized by HPLC. In order to estimate the amount of unreacted FM molecule, CMIPs were extracted with MeOH for quantifying the amount of reacted and unreacted FM molecules (shown in table 4). With this analysis, maximum binding sites (B_{max}) of CMIPs calculated. Among these CMIP1-9, the conditions used for preparing CMIP9 shows some promising results: having 45% (0.52 mmol) of FM molecule in 13 g of polymer particles results in an available B_{max} calculated as 40 µmole/g. The further recognition and selective studies have been conducted with CMIP9 and CNIP9.

3.4.4 REBINDING STUDIES

The B_{max} of 40 µmoles/g binding sites in CMIP9 are complementary to Fmoc-Cys-OH structural moieties that were able to recognize the corresponding template or structurally related templates through forming a disulfide bridge. In order to investigate the recognition properties, binding and selective studies were conducted by incubating different templates with different concentrations of templates of Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-*t*-Bu)-OH, Fmoc-Leu-OH, and Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$. The structures of four templates are shown in Figure 17. The recognition and binding of templates can be obtained by a disulfide exchange reaction between the tailing free thiol groups as binding sites and Fmoc-Cys(SH)-OH structural moieties. The chosen four templates all possess disulfide linkage in their structure except Fmoc-Leu-OH.

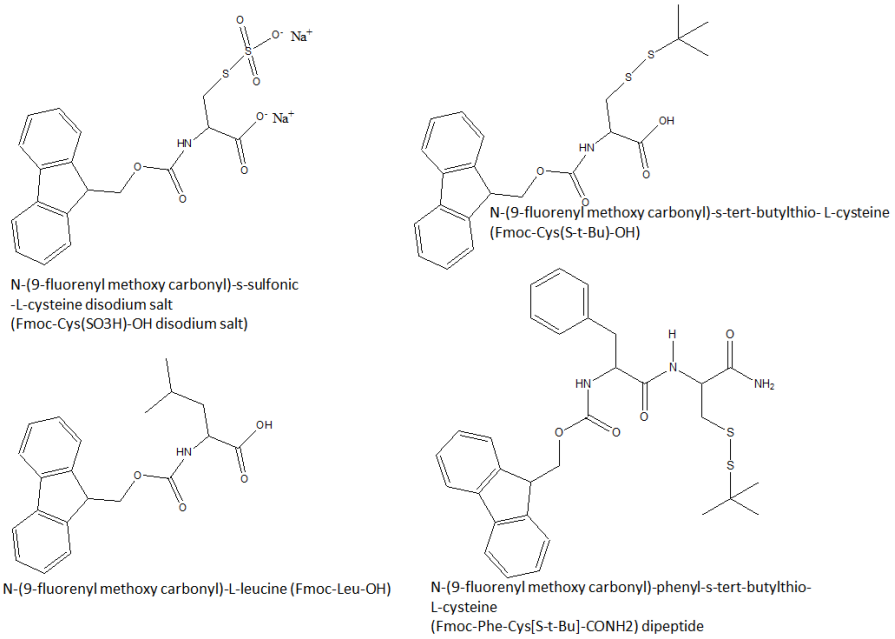


Figure 17: The structures of four templates used in rebinding and selective studies.

The dynamic nature of thiol-disulfide interconversion is more attractive and it can be controllable at certain pH conditions: at pH<5, disulfides are stable; they rapidly undergo interconversion into thiols at pH≥ 7 (Burri and Yu., 2015). The recognition and selectivity studies have been conducted in protic solvents (water and MeOH) at room temperature and at neutral pH. In order to confirm the spatial recognition, templates were designed by containing same moieties that could be complementary with binding sites of the CMIPs. To examine the selective recognition of Fmoc-Cys-

OH; a dipeptide compound (Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$) have been used. For the proof of concept, CNIPs were also designed to have a free tailing thiol group to facilitate the disulfide exchange reaction with the templates however without having Fmoc-Cys-OH moieties.

The results are shown in Figure 18; the templates Fmoc-Cys(SO_3H)-OH and Fmoc-Cys(S-*t*-Bu)-OH showing high binding affinities with CMIPs. Hence, that evidences that template recognition was carried out through thiol-disulfide exchange with the binding sites of CMIPs. The binding curves of CMIPs in the binding affinities are constantly saturating to 100% at each point (see Figure 18), proving that strong covalent recognition is a concentration independent manner with having same binding affinities in organic and inorganic solvents (Burri and Yu., 2015).

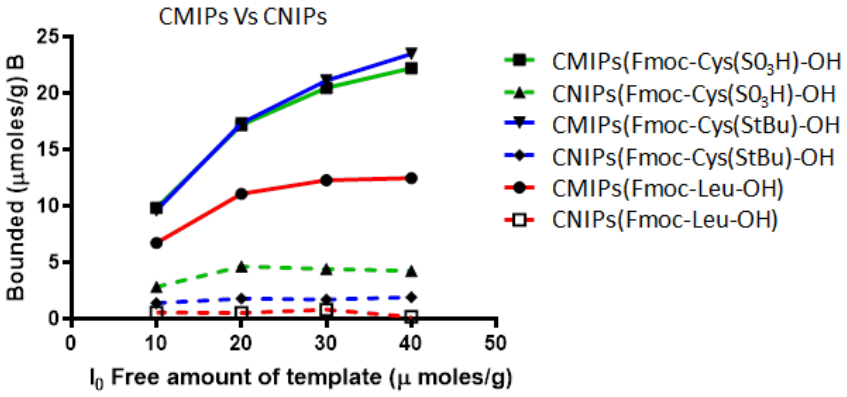


Figure 18: The recognition properties of CMIPs and CNIPs towards the templates Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-t-Bu)-OH and Fmoc-Leu-OH (Burri and Yu., 2015).

This could be more attractive over the non-covalent recognitions, because these non-covalent recognitions had been usually carried in organic solvents (the choice of solvents are limited) and concentration dependent, i.e., binding affinities various in different concentrations. Furthermore, the relative imprinting effect in CMIPs are inspected and compared with CNIPs. From Figure 18, we can conclude that the guest template molecules pronounce very poor binding with the CNIPs even though they contain the same functional groups which can be able to complete thiol-disulfide exchange with CNIPs. These results evidence that the template- mediated imprinting process creates shape specific moieties which are complementary to imprinting molecule.

3.4.5 COVALENT VS NON-COVALENT RECOGNITION

The guest template molecules recognition in CMIPs is based on the thiol-disulfide exchange principle, however Fmoc-Leu-OH was expected as non-covalent recognition. Since, Fmoc-Cys(SO_3H)-OH and Fmoc-Cys(S-*t*-Bu)-OH contains amides and hydroxyl functional groups in their structure that are able to form hydrogen bond interactions with the thiol groups located in the binding sites of MIPs. So the amount of bounded template is not only based on covalent recognition but may also due to such non-covalent recognition. Mukawa et al., (2003) reported that thiol groups in binding sites are able to form hydrogen bonding with the guest molecules. In order to examine this effect, the template bounded CMIP particles after batch rebinding experiments were extracted with MeOH and the obtained extracts were further analyzed and quantified on HPLC. We observed only 1-5% template liberated from CMIPs indicating that the rest of template 95-99% of amount was bounded with MIPs covalently (B_{cov}) and 1-5% amount bounded non-covalently ($B_{non\text{-}cov}$). For further proving this effect, the disulfide bonds in CMIP particles were reductively cleaved with $LiAlH_4$ and rebinding studies conducted under same manner again as described above. The repeated batch rebinding strengths (B_{rep}) relatively shows same binding strengths as B_{cov}. These results are proving the reproducibility of CMIPs. Figure 19 illustrates the flow chart of CMIPs reproducibility with Fmoc-Cys(S-*t*-Bu)-OH (Burri and Yu., 2015).

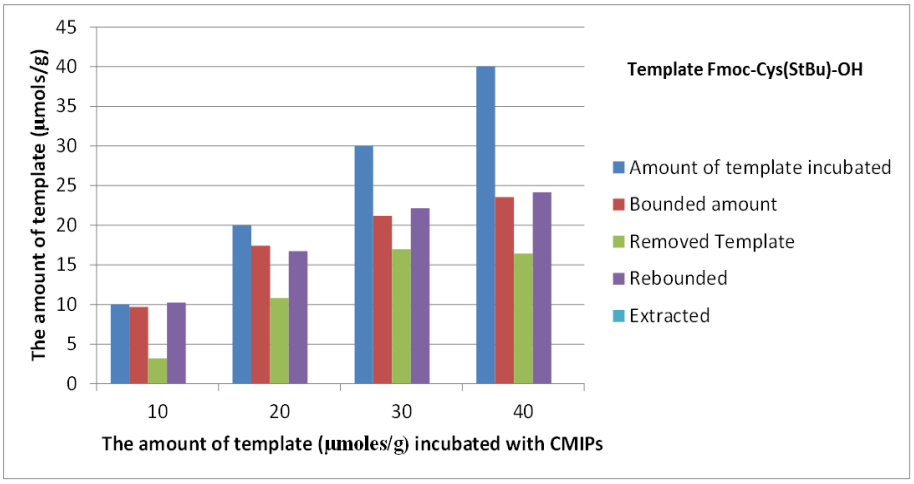


Figure 19: Flow chart of CMIPs reproducibility on the amount of template (Fmoc-Cys(S-t-Bu)-OH) incubated and correspondingly bounded, reduced, rebounded, and further extracted (Burri and Yu., 2015).

3.4.6 SELECTIVITY STUDIES

The relative selectivity of imprinted CMIPs were examined with template and its structurally related analogue molecules in CMIPs and CNIPs. The target template Fmoc-Cys(SO_3H)-OH and Fmoc-Leu-OH, Fmoc-Phe-Cys(S-*t*-Bu)-OH, and Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$ as structurally related analogues molecules were incubated with CMIPs and CNIPs with the same manner as above described rebinding experiments. In Figure 20, our results reveal the relative binding strengths for CMIPs towards various guest molecules which includes Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-*t*-Bu)-OH, Fmoc-Leu-OH, and Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$ (Burri and Yu., 2015).

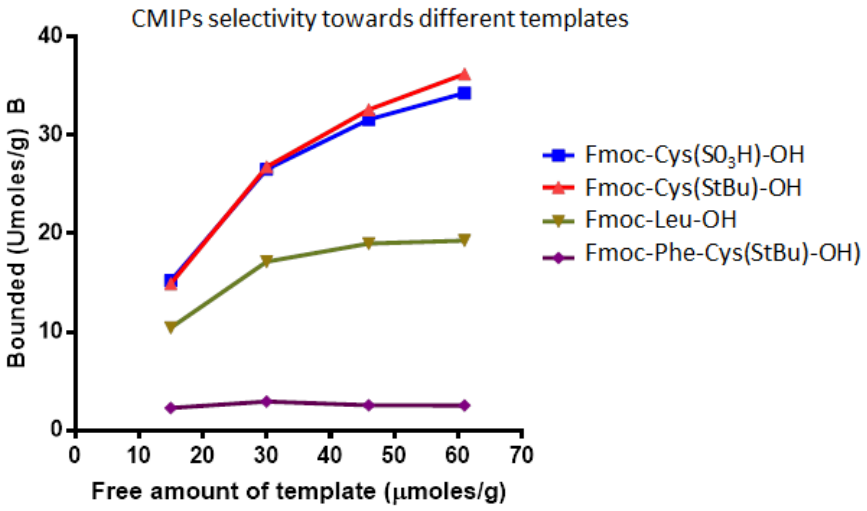


Figure 20: Relative binding strengths of CMIPs with various templates Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-t-Bu)-OH, Fmoc-Leu-OH, and Fmoc-Phe-Cys(S-t-Bu)-OH (Burri and Yu., 2015).

From Figure 18 and Figure 20; Fmoc-Leu-OH shows noticeably binding strengths towards CMIPs but not in CNIPs, indicating that Fmoc-Leu-OH non-covalently binding with CMIPs. Since CMIPs contains Fmoc-Leu-OH structure analogue moieties. Larger molecular moieties containing dipeptide Fmoc-Phe-Cys(S-*t*-Bu)-$CONH_2$ shows very poor binding affinities in both CMIPs and CNIPs (see Figure 20). These results strongly evidence that CMIPs are selectively recognizing the templates but not those template structure related to analogues of template from where CMIPs were derived.

3.5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, we have employed a novel synthetic strategy for covalent imprinting and recognition on the amino acids. The work demonstrated the feasibility of molecular recognition through reversible forming disulfide bonds through a dynamic thiol-disulfide interconversion. The results suggesting recognition of molecules (templates) takes places covalently, non-covalently (hydrogen bonding) in organic and inorganic solvents. However, rebinding experiments proved recognition with covalent interactions are effective and efficient capacities than non-covalent interactions. This nature of forming covalent bonds through reversible in imprinted MIPs can be reusable several times by reducing disulfides in templates without loss of molecular memory, which indicates MIPs can be reproducible with the same binding capacities that were maintained (Burri and Yu., 2015).

Additional studies will be performed with the synthetic cysteine receptor MIPs in ligand based assays in biological fluids to recognize the cysteine and their structurally related amino acids. Further to this; peptide receptor can be synthesized through disulfide founded imprinting and used as analytical reagents to recognize the relevant peptides in biological samples, therefore to check the related diseases. These sites directed thiol-disulfide exchange recognition model would be used in guessing the active sites of biological receptors and can be used in designing agonists and antagonists for receptor modulation (Burri and Yu., 2015).

CHAPTER. 4 IMPRINTING OF FMOC-PROTECTED CYSTEINE IN ORGANIC/INORGANIC HYBRIDS

4.1 MOTIVATION

The state-of-the-art in molecular imprinting is that the majority of the imprinted polymers of high activity and selectivity are successfully obtained and used, as long as the target molecules are Ångstrom-size and the recognition is achieved in organic media. However, the next theme for improving this technique is how to recognize nano-scaled large targets such as antiobiotics, peptide, or protein efficiently in water as natural receptors do. The imprinting process must be carried out also in water. However, only limited examples are reported so far (Andersson, 1996; Kugimiya et al., 1996) because of the following barriers: (1) hydrogen bondings, which are preferentially used for the pre-organization of templates and functional monomers, are easily destroyed in water due to competition with the solvent (Allender et al., 1999), (2) the imprinted polymer is not stiff and thermal stable enough for certain type of applications (Haupt and Mosbach, 2000).

These above-mentioned problems can be overcome by a combination of inorganic and organic phases within the materials, i.e., inorganic/organic hybrid material (Novak and Bruce, 1993). A combined property of glasses and organic polymers, like high mechanical strength, good optical transparency, chemical inertness, high stability and negligible swelling in aqueous and organic solvents, can be obtained. By choosing such model compounds as sugars, oligosaccharides, and peptides as target template molecules, cheap and fast feasible analyses for evaluating the imprinting effects will be built up. Therefore, these magnificent properties of hybrid materials caught an increasing interest in the possibility of producing advanced molecularly imprinted hybrid polymers.

The molecularly imprinted hybrid materials are generally prepared via sol-gel process followed by radical polymerization. The most attractive features of sol-gel process are that it favors low temperatures (ambient), allowing organic polymers to be incorporated throughout porous silica network. In this project, we have synthesized the six hybrid materials without using templates for finding optimal conditions for preparing hybrid materials. With these findings on the optimal parameters, we imprinted N-(9-fluorenyl methoxy carbonyl)-L-cysteine (Fmoc-Cys-OH) in such organic/inorganic hybrid materials. Consequently, imprinted hybrids recognition properties, binding strengths and selectivity have been tested. The printed molecule (9-fluorenyl methoxy carbonyl)-S-(prop-2-ene-1-thiol)-L-Cysteine (Fmoc-

Cys(SCH_2CHCH_2)-OH **(5)** was obtained as compound **(5)** from the above mentioned experiments in Chapter-3. The organic polymer network was then obtained by co-polymerizing the crosslinker (EGDMA) with the functional monomer (imprinted template molecule) within the already made inorganic silica network which had been obtained through sol-gel derived triethoxyorthosilicate (TEOS). Control hybrid polymers were prepared with the same manner by replacing FM molecule with (3-mercaptopropyl)triethoxysilane (TESH).

4.2 MATERIALS AND EXPERIMENTAL PROCEDURES

The materials and instrumentations used for preparing organic polymeric networks described in Chapter-3 are applied in this work, too. Addition to this, triethoxyorthosilicate (TEOS), methyl methacrylate (MAA),28% ammonium hydroxide solution and (3-mercaptopropyl)triethoxysilane (TESH) were purchased from Sigma-Aldrich GmBH (Germany). Acetone was ordered from Prolabo (VWR) and ethanol (EtOH) from Kemetyl A/S. Thermogravimetric analysis was performed on STA 449C Thermogravimetric analyzer from NETZSCH Germany. The Fmoc-Cys(SCH2CHCH2)-OH (5) as functional monomer was directly used from the previously prepared ones (Described in Chapter-2).

4.2.1 PREPARATION OF ORGANIC/INORGANIC HYBRIDS

Three types of hybrid materials were prepared via sol-gel process followed by radical polymerization. In preparation of first type hybrid polymers (H1 to H6), TEOS was used as inorganic precursor and MAA was chosen as organic monomer. In a 50 mL round bottom flask connected with condenser, TEOS, MAA, crosslinker (EGDMA), initiator (AIBN), and half amount of EtOH (solvent) were mixed together at 22 ^oC. The reaction mixture was heated to 60 ^oC and then base catalyst (28% ammonium hydroxide solution) and remaining half amount of EtOH was added to reaction mixture. The sol-gel reaction was kept for 20 h. Afterwards solvents and water was removed from the reaction mixture by rotary evaporation. Radical polymerization was initiated and preceded in remaining materials at 80 ^oC for 24 h. After completion of radical polymerization, bulky hybrid materials were grinded in to fine particles. The amounts of all components and molar ratios for hybrid products are given in Table 5.

The second type of hybrid materials (HMIP1 and HMIP2) prepared via template mediated molecularly imprinting procedure. The experiments were carried out in the same procedure as above mentioned. However, Fmoc-Cys(SCH_2CHCH_2)-OH **(5)** was used as organic precursor (template) instead of MAA. The amounts of all components and molar ratios are listed in Table 6.

The third type of hybrid materials (HNIP1 and HNIP2) are the control polymers prepared in the same as above mentioned procedure. However, prop-2-ene-1-thiol was

used as organic precursor (template) instead of Fmoc-Cys(SCH_2CHCH_2)-OH **(5)**. The amounts of all components and molar ratios are shown in Table 6.

4.2.2 SOXHLET EXTRACTION

The grinded polymeric hybrid materials are packed in a microfiber filter (6µM meshes) and placed in soxhlet extractor. The materials were extracted with acetone for 24 h. The particles extracted with acetone were denoted with H7- H12. The hybrid particles without extraction were named with H1-H6. All hybrid samples (H1- H6 and H7-H12) were analyzed on thermogravimetric analyzer (TGA)in temperature ranges from 50 to 550 ºC at a heating rate of 20ºC/min.

The imprinted and control hybrids HMIP1, HMIP2, HNIP1, and HNIP2 were extracted with MeOH to remove unreacted monomers, crosslinkers and certain oligomers.

4.2.3 TEMPLATE REMOVAL

Disulfide bonds in the polymeric hybrid particles (HMIP1 and HMIP2) were reductively cleaved with reducing agents. In a round bottomed flask, polymer particles were suspended in 2M $LiAlH_4$ in THF solution. The reaction mixture was stirred for 18 h at room temperature. Further diluted sulfuric acid and methanol was added dropwise to reaction mixture. Polymer particles were filtered off and supernatant was analyzed on HPLC.

4.2.4 REBINDING STUDIES

Batch rebinding and selectivity experiments were carried out in water for Fmoc-Cys(SO_3H)-OH, and in methanol for the other guests. In a series of 50 mL centrifuge tubes contained certain amount of HMIPs and HNIPs were incubated with known amount (10, 20, 30 and 40 µmole) of various templates which includes Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-*t*-Bu)-OH, and Fmoc-Leu-OH, respectively. The chemical structures of four templates were shown in Figure 17. The reaction tubes were placed on shaking table for 18 h, and then centrifuged; the supernatants were analyzed on HPLC. With our previous results we believed that short incubation periods are enough for templates' binding in covalently imprinted MIPs. To confirm this, HMIP2 hybrid polymers were separately incubated with Fmoc-Cys(SO_3H)-OH and Fmoc-Leu-OH for 5 h. The binding affinities were calculated in regular time intervals.

4.3 RESULTS AND DISCUSSION

4.3.1 HYBRID MATERIALS

Three types of hybrid materials were synthesized with the sol-gel process followed by radical polymerization. In table 5, the amounts of various components used in hybrid materials synthesis are presented. The first type of hybrid materials was synthesized for optimization of synthetic process without using templates, and denoted with H1-H6. Some portion of these hybrid materials were washed (soxhlate extraction) and denoted as H7- H12, corresponding to their own non-extracted ones from H1- H6, respectively. This means that H1, H3, and H5 are all composed of interpenetrating organic/inorganic networks, while only linear polymer PMMA were embedded in the silica network of H2, H4, and H6, when PMMA was not crosslinked. Meanwhile, different molar ratios of organic and inorganic components had been employed in hybrids synthesis: H1/H2 has an equal amount between organic and inorganic components; H3/H4 contains about twice amount SiO_2 of PMMA; in H5/H6, the organic content PMMA is two times of inorganic SiO_2. Washing of hybrids H7– H12 with acetone was to remove the unreacted components, dissolve the low molecular weighted organic polymers and the organic content which was not highly interpenetrated with inorganic network (Deng et al., 2004). Since the organic initiator AIBN was used in very low concentration, it has not been taken in to account in calculating organic/inorganic ratios, otherwise the ratios could be slightly varied.

Table 5: The amounts of different components and the ratios between organic and inorganic contents of hybrid materials synthesis. (Burri, 2009; Nielsen, 2009).

Hybrid	**TEOS (mmol)**	**EtOH (mmol)**	**NH_4OH 28% (mL)**	**MMA (mmol)**	**EGDMA (mmol)**	**AIBN (mg)**	**Organic/inorganic (mole/mole) ratio**
H1	15.56	33.38	0.80	7.78	7.78	128	1.00
H2	15.56	33.38	0.80	15.56	0	128	1.00
H3	21.78	46.22	1.12	4.67	4.67	77	0.43
H4	21.78	46.22	1.12	9.34	0	77	0.43
H5	9.21	19.86	0.475	10.89	10.89	177	2.36
H6	9.21	19.86	0.475	21.78	0	177	2.36

4.3.2 THERMOGRAVIMETRIC ANALYSES

The thermogravimetric Analyses (TGA) results are shown in Figure 21-23. The hybrid materials H1 and H2 contain the equal portions of organic and inorganic content. However when preparing H1 materials, equal portions of MAA and EGDMA had been utilized as organic content; but in preparing H2 materials, only MAA was adopted without any crosslinking reagents. The TGA analysis shows the weight loss of H1 and H7 materials 41.5% and 49% respectively.

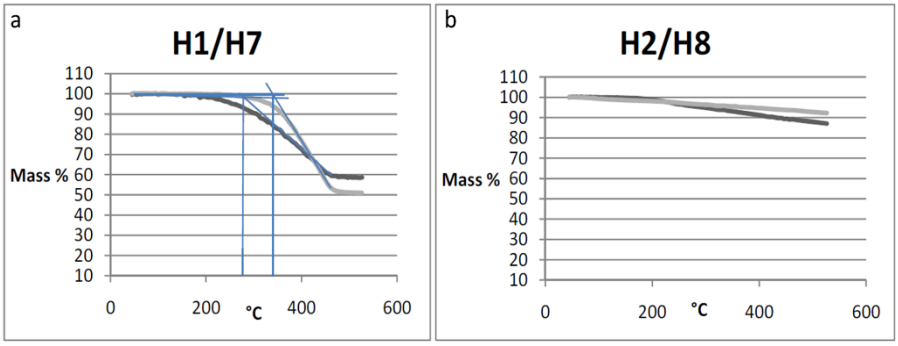


Figure 21: TGA results of the hybrid materials H1 and H7 (a) and H2 and H8 (b). The pale gray color line represents the hybrid materials, which have been washed with acetone (H7 and H8); the dark grey color represents the hybrid materials which have not been wash (H1 and H2). The decomposition temperatures estimated with extrapolated onset lines (Burri, 2009; Nielsen, 2009).

TGA results of the hybrid materials H1 and H7 (a) and H2 and H8 (b). The pale gray color line represents the hybrid materials, which have been washed with acetone (H7 and H8); the dark grey color represents the hybrid materials which have not been washed The weight loss of H2 and H8 materials is 12.9% and 7.8% (Figure 21), respectively. These results suggest methanol extraction have successfully removed the unreacted organic content, dimers, trimers & oligomers of organic components and some part of organic content which have been not penetrated well in inorganic network. Thermal treatment can get these impurities removed in a comparable manner as solvent extraction.

The hybrid materials H3 and H4 prepared with same organic/inorganic ratio, but with and without the crosslinking. This means that H3 are composed of interpenetrating organic/inorganic networks, while only linear polymer PMMA were embedded in the silica network of H4. Figure 22 expresses the results of weight loss of 37.9%, 39.9%, 22.9%, and 7.5% for H3, H4, H9, and H10, respectively.

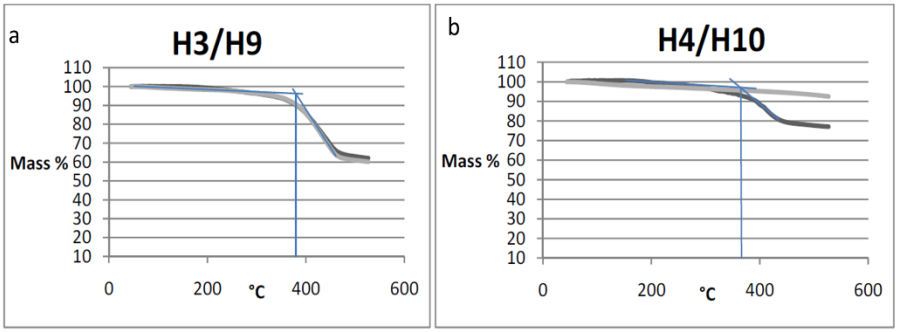


Figure 22: TGA results of the hybrid materials H3 and H9 (a) and H4 and H10 (b). The pale gray color line represents the hybrid materials which have been washed with acetone (H9 and H10) and the dark grey color represents the hybrid materials which have not been washed (H3 and H4). The decomposition temperatures estimated with extrapolated onset lines (Burri, 2009; Nielsen, 2009).

Figure 23 exhibit the results of H5, H6, H11 and H12. These hybrids (H5 and H6) prepared under same organic/inorganic ratios however differ in amounts of cross linking used. H5 and H11 pronounce weight loss of 83% and 83.2 % due to high content of organic network. And H6 and H12 reveal the weight loss of 79.7% and 7% respectively. These results prove that wash of the materials shows the great loss of organic network, on the other hand the washing step doesn't show any effect on H5 and H11. In general hybrid materials without crosslinker present great loss in organic content. Conversely these results do not follow the same tendency; this indicates that prepared hybrid materials contain too high molar mass of organic content or too highly interpenetrated with inorganic networks.

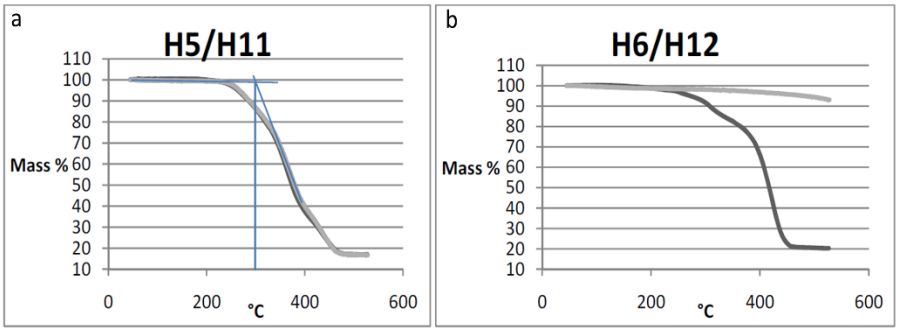


Figure 23: TGA results of the hybrid materials H5 and H11 (a) and H6 and H12 (b). The pale gray color line represents the hybrid materials which have been washed with acetone (H11 and H12) and the dark grey color represents the hybrid materials which have not been washed (H5 and H6). The decomposition temperatures estimated with extrapolated onset lines (Burri, 2009; Nielsen, 2009).

Based on these results; we have set parameters for imprinting Fmoc protected cysteine. However, too dense inorganic network block templates transformation hence used high organic content hybrid materials.

4.3.3 IMPRINTED HYBRID MATERIALS

As mentioned above; the second type materials (imprinted hybrids HMIP) and third type of materials (non-imprinted hybrids HNIP) have been synthesized by using sol-gel process followed by radical polymerization. The amounts of components and ratios are summed-up in table 6. The molar ratios of organic and inorganic components 3:1 and 4:1 were employed in HMIP1/HNIP1 and HMIP2/HNIP2 respectively. The synthesized hybrid materials were washed with methanol to remove unreacted components and reaction intermediates. The supernatant was applied on HPLC so that the reacted and unreacted amount of FM was quantified.

In order to remove the template molecules from the imprinted polymers, HMIP1 and HMIP2 were suspended in 2M $LiAlH_4$ in THF solution and stirred for 18 h, further the supernatant was quantified on HPLC and amount of template cleaved from the polymers were quantified with the standard curves of FM. The available maximum binding sites were denoted with B_{max}.

*Table 6: The compositions and ratios of organic and inorganic contents for imprinted hybrid materials. Functional monomer Fmoc-Cys(SCH_2CHCH_2)-OH **(5) was** used as organic precursor along with EGDMA. However prop-2-ene-1-thiol was used as monomer in HNIP1 and HNIP2.*

Hybrid	**FM (mmol)**	**EGDMA (mmol)**	**TEOS (mmol)**	**THF (mmol)**	**EtOH (mmol)**	**NH_4OH (mL)**	**AIBN (mg)**	**O/I molar ratio**
HMIP 1	1	10	3.66	200	7.75	0.5	128	3
HMIP 2	1	10	2.75	200	7.75	0.5	128	4
HNIP 1	1	10	3.66	200	7.75	0.5	128	3
HNIP 2	1	10	2.75	200	7.75	0.5	128	4

B_{max} is defined as $Bmax = \frac{n\ templates}{m\ CIS}$ (Yan and Ramstrom, 2005). The amount of templates released from the HMIPs was given in Table 7. The reduced amount of template from the polymers is very low comparing to the reacted amount of FM i.e. 15% and 29% in HMIP1 and HMIP2 respectively. These results are expected due to high rigidity of the highly densed inorganic network content in HMIP2.

Table 7: The amounts of initial amount of FM (I_{FM}), unreacted FM (UR_{FM}), reacted FM (R_{FM}), mass of hybrids, released template amount and available maximum binding sites in hybrids.

Polymer	**I_{FM} mmol**	**UR_{FM} mmol**	**R_{FM} mmol**	**Total mass of Hybrids (g)**	**Amount of template released mmol (µmol)**	**Bmax mmol (µmol/g)**
HMIP1	1	0.1	0.9	2.7	0.142 (142)	0.052 (52)
HMIP 2	1	0.6	0.94	5.0	0.273 (273)	0.054 (54)

4.3.4 REBINDING STUDIES

As described in Chapter-3, to investigate the recognition properties of hybrid materials, batch rebinding studies were conducted. The HMIPs and HNIPs were incubated with three different template molecules with known concentrations. The structures of used templates, Fmoc-Cys(SO_3H)-OH, Fmoc-Cys(S-*t*-Bu)-OH, and Fmoc-Leu-OH were shown in Figure 17. The results of rebinding strengths of templates with HMIP1 and HMIP2 are presented in Figure 24a and 24b. The template Fmoc-Cys(SO_3H)-OH exhibits good binding affinities towards HMIPs. However, amount of bonded template is higher than the free available binding sites in HMIPs. In HMIP1 the maximum available binding sites is 52 µmoles/g, nevertheless, at higher amounts templates incubation pronounces a higher amount of template binding (62 µmoles/g) than the available binding sites (52µmoles/g).

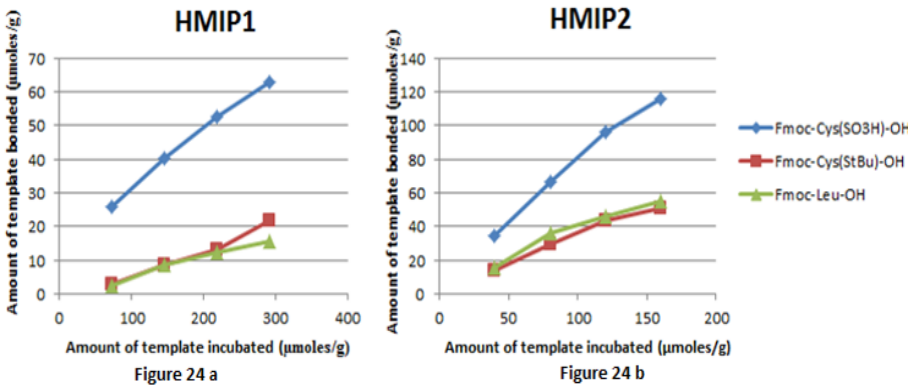


Figure 24: Binding strengths of three different structurally related templates (Fmoc-Cys(SO3H)-OH, Fmoc-Cys(S-t-Bu)-OH and Fmoc-Leu-OH in HMIP1 (24a) and HMIP2 (24b).

In the same manner, in HMIP2 the maximum available binding sites is 54 µmoles/g, but the higher amount of template binding (120 µmoles/g) than the available B_{max} had been found. Any excessive rebinding observed beyond this point could be the unspecific template interactions with the polymer networks due to their porous nature. On the other hand, the maximum binding sites had been calculated based on the found amount of templates in the supernatants, which were cleaved from the hybrids. This could have been under-estimated, if some of removed templates got remained inside the networks due to its high degree of crosslinking density.

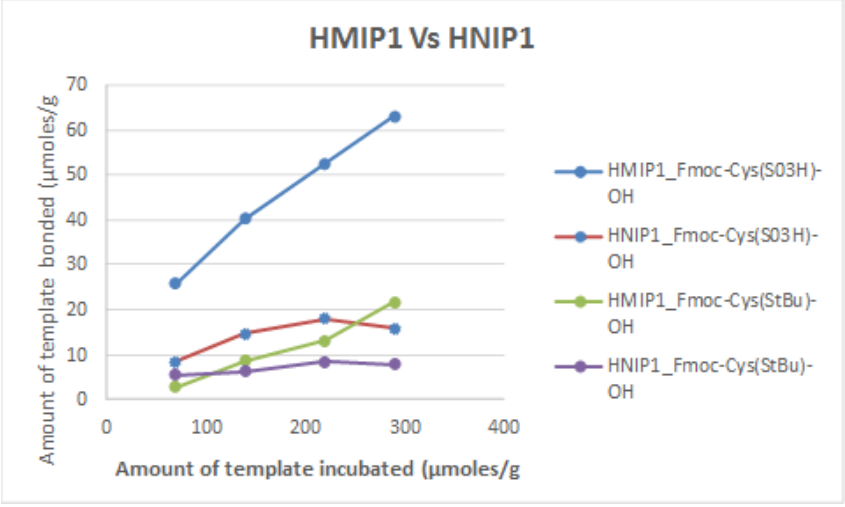


Figure 25: : Relative binding strengths of Fmoc-Cys(SO_3H)-OH and Fmoc-Cys(S-t-Bu)-OH in HMIP1 and HNIP1.

However, the other templates Fmoc-Cys(S-*t*-Bu)-OH and Fmoc-Leu-OH are showing reasonable binding strengths corresponding with their maximun number of binding sites. Since Fmoc-Cys(S-*t*-Bu)-OH binding strategy is similar to Fmoc-Cys(SO_3H)-OH by covalently reforming disulfide bond via thiol-disulfide exchange reaction. Conversely, Fmoc-Leu-OH was expected to be bound via non-covalent binding.

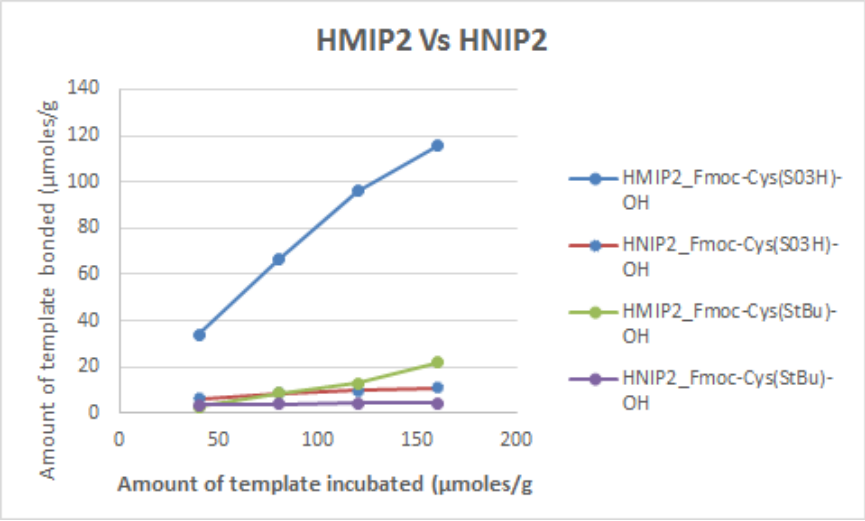


Figure 26: Relative binding strengths of Fmoc-Cys(SO3H)-OH and Fmoc-Cys(S-t-Bu)-OH in HMIP2 and HNIP2.

In order to investigate the non-specific binding; rebinding experiments were performed in non-imprinted hybrids (HNIPs). Figure 25 and Figure 26 present comparatively the relative binding strengths of templates for HMIPS and HNIPs. Both HMIP1 and HMIP2 pronounces higher binding affinities than HNIP1 does towards Fmoc-Cys(SO_3H)-OH, indicating reasonably good imprinting effects. The much lower rebinding towards more steric hindered template Fmoc-Cys(S-*t*-Bu)-OH of both two MIPs presents good rebinding selectivity, too. Fmoc-Cys(SO_3H)-OH and Fmoc-Cys(S-*t*-Bu)-OH binding strategy is straight forwarded that they were binded covalently via disulfide exchange with the MIPs. Fmoc-Leu-OH binding with non-covalent interactions with polymers was expected. Fmoc-Leu-OH also presented certain binding affinities to HMIP1 and HMIP2. It suggests that HMIPs recognize the structurally related templates via both covalent and non-covalent interactions.

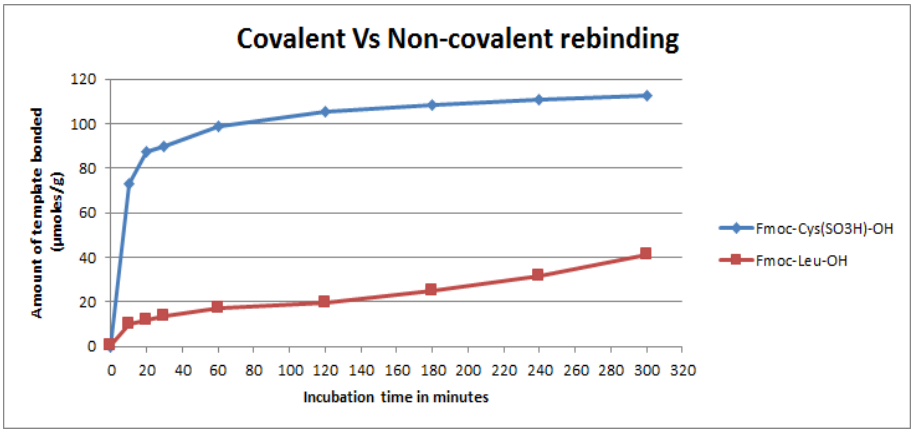


Figure 27: Comparison between covalent and non-covalent binding affinities respective with incubation time on HMIP2.

As our previous results revealed, short incubation times are enough for covalent rebinding of the templates. In order to investigate the time dependence on covalent and non-covalent binding affinities, HMIP2 particles were separately incubated with Fmoc-Cys(SO_3H)-OH and Fmoc-Leu-OH and the bonded amount of template was measured under different time intervals. The binding results of covalently bonded [Fmoc-Cys(SO_3H)-OH] and non-covalently bound [Fmoc-Leu-OH] are shown in Figure 27. In shorter incubation periods (0-10 mints), most of the template (Fmoc-Cys(SO_3H)-OH was bonded covalently with the HMIPs and Fmoc-Leu-OH recognition carried out non-covalently with a much slower kinetics, when comparing to the covalent manner. These results prove that such covalent rebinding via disulfide bonding is specific and fast (HPLC chromatograms presented in Appendix D).

4.4 CONCLUSIONS AND PERSPECTIVES

Three types of hybrid materials have been successfully synthesized using sol-gel process followed by radical polymerization. The hybrid materials were produced by applying various compositions of organic/inorganic contents. Thermogravimetric analyses was performed on first type of hybrid materials and showed that the organic content decomposed between 270 OC - 375 OC. Hybrid materials which were produced without using crosslinkers showed great mass loss in organic content, hence these hybrid materials are not highly interpenetrated with the inorganic network.

The second and third type of hybrid materials were produced for preparing covalent imprinting with disulfides in hybrid materials. A novel silica based organic synthetic hybrid receptor Fmoc-Cys-OH was prepared by sol-gel processing with tetramethoxy orthosilicate (TEOS) in presence of Fmoc-Cys(SCH_2CHCH_2)-OH) followed by crosslinking with ethylene glycol dimethacrylate (EGDMA). Subsequently disulfides

reductively cleaved, resulted thiol containing hybrid polymers recognized selectively Fmoc-Cys(SH)-OH in water with high binding affinity. Constant binding affinities were observed in batch rebinding experiments followed by disulfide reductions, strongly indicating that imprinted polymers can be reused. The covalent and non-covalent binding kinetics suggested that templates were covalently bonded to imprinted polymers in an efficiently short period of time. We found the attractive high binding recognition of sulfur containing cysteine covalently (thiol-disulfide exchange) with high selectivity in water, and this could be used to develop models to recognize proteins in biological samples which contain cysteine residues in their structure.

CHAPTER 5. IMPRINTING OF BENZYL MERCAPTAN IN SILICA NETWORK

5.1 MOTIVATION

So far, molecularly imprinted polymers have been successfully prepared in organic polymers through radical polymerization via covalent bindings and result in efficient template recognition carried out in organic- and aqueous-media. Nevertheless, there are limited reports available on molecular imprinting in inorganic materials and even less covalent imprinting approach have been applied. Molecular imprinting in inorganic materials can be more attractive over organic ones, since these rigid inorganic polymeric materials present good mechanical, thermal, and optical properties and negligible swelling in mobile phases. These smart features of inorganic materials are attractive to prepare novel imprinted polymers. In addition, covalent imprinting with disulfides can be performed at even higher temperature while sol-gel process can be performed at 55 ºC. Chemical inertness of silica materials allows wide range of chemicals and reactions for removal of imprinted template and even for high temperature (>500 ºC) process (burning templates off).

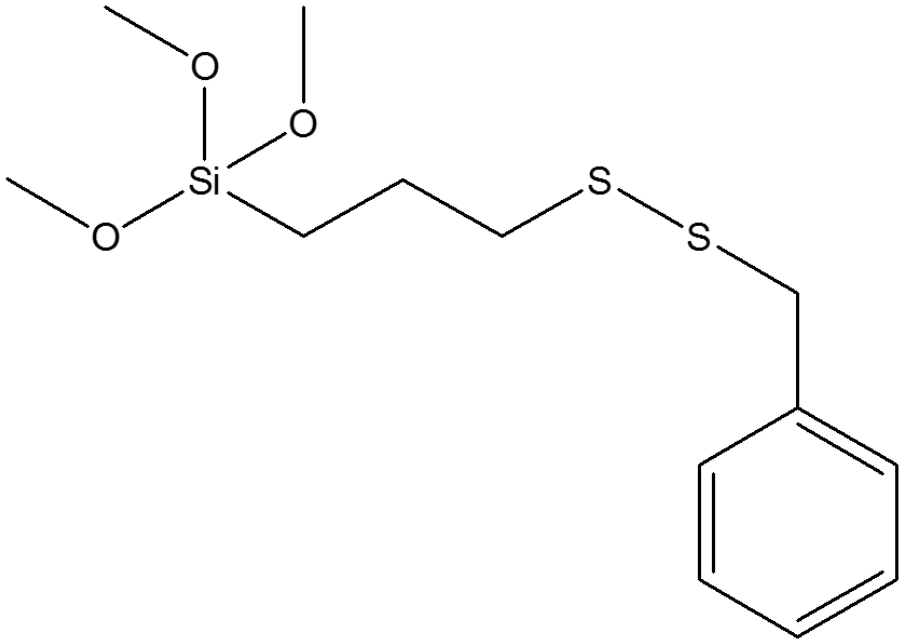


Figure 28: Structure of functional monomer (FM) (3-benzyldisulfanylpropyl trimethoxysilane).

With these attractive features of imprinted silica network, imprinting of benzyl mercaptan like synthetic receptors was targeted. The functional monomer (FM) 3-benzyldisulfanylpropyl trimethoxysilane was designed, synthesized, and characterized. The structure is shown in Figure 28, while the template (benzyl mercaptan) was coupled with functional monomer (3-mercaptopropyl) trimethoxysilane through a disulfide linkage.

5.2 MATERIALS AND EXPERIMENTAL PROCEDURES

The materials and instrumentations used for preparing sol-gel processed inorganic silica networks are not instructed when they are the same ones as those described in Chapter-2 and Chapter-4. Templates/guest molecules for batch rebinding and selective studies used are the same products from previous project (Chapter-2). However, phenol was replaced by benzyl alcohol.

5.2.1 FUNCTIONAL MONOMER

As described in Chapter-3, for preparation of 3-benzyldisufanylprop-1-ene, the same synthetic steps as that for 3-benzyldisufanylprop-1-ene were followed. The synthetic procedure consists 3 steps of reactions. In step-1, thiolate form of (3-mercaptopropyl) trimethoxysilane was prepared by adding 50 mmol (5.56 mL) of (3-mercaptopropyl) trimethoxysilane to 30 mmol (1.62 g) of sodium methoxide in 10 mL of methanol; in step-2, sodium benzyl thiosulfate was prepared; In step-3, thiolate form of (3-mercaptopropyl) trimethoxysilane solution was mixed with 30 mmol (6.8 g) of sodium benzyl thiosulfate in 150 mL of methanol at room temperature. After 1 h period, methanol was removed from reaction mixture under rotary evaporator. The byproduct sodium sulfite was removed by adding (2×200 mL) diethyl ether, further filtration and evaporation of diethyl ether gave 8.2 g (26 mmol) of 3-benzyldisulfanylpropyl trimethoxysilane as oil product with 78% yield. The NMR spectra presented in Appendix C. NMR: ^{1}H NMR (600 MHz, DMSO-d_6, δ): 7.30 (5H, Ar H), 3.87 (2H, CH_2), 3.55 (9H, CH_3), 2.39 (2H, CH_2), 1.7 (2H, CH_2), 0.72 (2H, CH_2) (Burri, 2010; Nielsen, 2010).

Preparation of sodium benzyl thiosulfate and sodium tertiary-butyl benzyl thiosulfate were also carried out in the same procedure as described in Chapter-2, section 2.3.1.2.

5.2.2 PREPARATION OF INORGANIC POLYMERS THROUGH SOL-GEL PROCESS

Preparation of inorganic silica polymers through sol-gel processing was described in Chapter-4. The amount of chemicals, solvents, catalysts, and reaction times employed in this synthesis are listed in Table 7. For a typical synthesis procedure of imprinted silica particles (CIS1): in a 50 mL round bottom flask connected with condenser,

functional monomer [(3-benzyldisulfanylpropyl)trimethoxysilane], cross linker (TEOS), and half amount of EtOH (solvent) were mixed at 22 ^{o}C. The reaction mixture was heated to 60 ^{o}C, followed by addition of a base catalyst (28% ammonium hydroxide solution) and remaining half amount of EtOH. The sol-gel reaction was kept running for 20 h. Afterwards solvents and water were removed from the reaction mixture by rotary evaporation. The resulted polymeric particles were grinded in to fine particles. The non-imprinted of silica particles or control polymers (NIS) were prepared under the same procedure as above mentioned. However, functional monomer 3-benzyldisulfanylpropyl trimethoxysilane was replaced by (3-mercaptopropyl) trimethoxysilane. (Burri, 2010; Nielsen, 2010).

The resultant CIS and NIS polymer particles were washed with methanol for 18 h to remove the unreacted components and any byproducts. The washing was monitored by analyzing the supernatant on HPLC until getting rid of all unreacted components. The amount of unreacted functional monomer in CIS was estimated with quantifying the washed supernatants (Burri, 2010; Nielsen, 2010).

5.2.3 TEMPLATE REMOVAL

Disulfide bonds in the imprinted silica particles (CIS) were reductively cleaved with reducing agents. Certain amount of polymer particles were suspended in sodium borohydrate $NaBH_4$ in methanol solution. The reaction mixture was kept stirring for 18 h at room temperature, followed by separation of solid and liquid phases via filtration (Burri, 2010; Nielsen, 2010; Burri and Yu, 2016).

5.2.4 POST MODIFICATION ON CIS AND NIS

The novel post modification was employed for the conversion of thiolic binding sites to thiolated binding sites without altering the shape and other physical properties of binding sites. The certain amount of polymer particles (CIS/NIS) were suspended in anhydrous acetonitrile. Then excess (2 -fold excess to amount of polymer particles) amount of sodium hydride was added to the reaction mixture and kept reacting for 18 h at 22 ^{o}C to convert the free thiols (-SH) in binding sites to sodium thiolated form (-$S^{-}Na^{+}$) (Burri, 2010; Nielsen, 2010; Burri and Yu, 2016).

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5.2.5 BINDING AND SELECTIVITY STUDIES

Batch rebinding and selectivity experiments were carried out in methanol. The four different templates used in batch rebinding and selective studies were sodium benzyl thiosulfate, sodium tertiary benzyl thiosulfate, benzyl alcohol, and benzyl mercaptan. The standard curves of four templates were prepared based on HPLC analysis. In a

series of 50 mL centrifuge tubes contained CIS and NIS were incubated with known concentration of templates. Then reactions tubes were placed on shaking table for 18 h, followed by centrifugation, then the supernatant was analyzed on HPLC (Burri, 2010; Nielsen, 2010).

5.3 RESULTS AND DISCUSSION

5.3.1 FUNCTIONAL MONOMER

The functional monomer (FM) [(3-benzyldisulfanylpropyl)trimethoxysilane] prepared from template (benzyl mercaptan) and [(3-mercaptopropyl) trimethoxysilane] were all covalently coupled with silica precursor via a disulfide bond. The structure and NMR spectra of FMs are shown in Figure 28 and Appendix C respectively. FM has versatile uses in imprinting: 1.) The main advantage of these covalently coupled template and functional monomer in 1:1 molar ratios increase homogeneity of the binding sites, as well as distribute binding sites throughout polymeric particles; 2.) The metal alkoxides (trimethoxy silicates) can formed silica network through sol-gel processing; 3.) Sol-gel process was performed at high temperatures (60 ^{O}C), hence covalent imprinting (with disulfide bunds) is best suitable for this purpose; and 4.) Disulfide bonds facilitate easy removal of template from the imprinted polymers as well as useful in recognition process through reversibly forming thiol-disulfide exchange reaction.

5.3.2 IMPRINTED AND NON-IMPRINTED POLYMERS FROM SOL-GEL PROCESS

Very few limited reports were available describing molecular imprinting for sol-gel processed inorganic silica network and optimal parameters haven't been well reported yet, let alone disulfide based imprinting and recognition. With previously reported results (Larsen, 2006) and our results in Chapter-2 and Chapter-4, we have set optimal parameters for covalent imprinting procedure. Ethanol used as solvent because FM, cross linkers (TEOS), and reaction intermediates are finely soluble in solvent (Brinker and Scherer, 1990). As reported (Nielsen, 2009), particle sizes and their distribution influence greatly on altering reaction parameters. With these evaluations, we have prepared numerous imprinted and non-imprinted polymers. The molar amounts of chemicals and solvents that are employed in sol-gel processing are listed in Table 8.

Table 8: The components and ratios between water and inorganic precursors of sol-gel silica materials synthesis. [(3-benzyldisulfanylpropyl)trimethoxysilane] and [(3-mercaptopropyl) trimethoxysilane] were used as functional monomer for n imprinted polymers (CIS) and non-imprinted polymers (NIS), respectively (Burri, 2010; Nielsen, 2010).

Polymers	**FM (mmol)**	**TEOS (mmol)**	**EtOH (mmol)**	**NH_3 (mmol)**	**H_2O (mL)**	**R Valuve (H_2O:Precursor)**
CISa	10	0	37.7	16.7	81.2	8.12
CISb	10	2.5	37.7	16.7	81.2	6.5
CISc	10	5.0	37.7	16.7	81.2	5.41
CISd	10	7.5	37.7	16.7	81.2	4.64
CIS1	10	10	37.7	16.7	81.2	4.06
CIS2	10	20	37.7	16.7	81.2	2.71
CIS3	10	30	37.7	16.7	81.2	2.03
NISa	10	0	37.7	16.7	81.2	8.12
NISb	10	2.5	37.7	16.7	81.2	6.5
NISc	10	5.0	37.7	16.7	81.2	5.41
NISd	10	7.5	37.7	16.7	81.2	4.64
NIS1	10	10	37.7	16.7	81.2	4.06
NIS2	10	20	37.7	16.7	81.2	2.71
NIS3	10	30	37.7	16.7	81.2	2.03

5.3.3 REMOVAL OF TEMPLATES AND CREATION OF BINDING SITES

The imprinted polymers were suspended in sodium borohydride methanolic solutions, the supernatant was analyzed on HPLC, and amount of template cleaved from the polymers were quantified with the standard curves of benzyl marcaptan. The maxium binding sites is denoted as B_{max}, i.e., $Bmax = \frac{n\ templates}{m\ CIS}$ (Yan and Ramstrom, 2005). Polymers CISa, CISb, CISc and CISd did not pronounce any of template removal from the polymers, due to the very less amounts of crosslinker (TEOS) employed. Therefore, FM was washed out in the washing procedure on polymers. Hence, optimal crosslinking has not been taken place, this resulted in losing the unreacted or partially crosslinked templates during the washing steps of the polymers with methanol. However, in CIS1, CIS2, and CIS3 the amount of crosslinkers employed were 1, 2, and 3 times more than the amount of FM employed. Therefore, the dense crosslinked network was formed, leading to comparatively less amount template released from the CIS3. Table 9 finds the amounts of templates released and maximum binding sites, B_{max}, distributed in CIS.

Table 9: The total mass of CIS, amounts of template release and maximum available binding sites distributed throughout the CIS (Burri, 2010; Nielsen, 2010).

Polymer	**Total mass of CIS (g)**	**Template release (mmol)**	**B_{max} (mmol/g)**
CIS1	1.61	0.699	0.434
CIS2	2.79	1.58	0.566
CIS3	4.08	0.198	0.0485

5.3.4 BATCH REBINDING AND SELECTIVE STUDIES

Batch rebinding and selectivity studies have been conducted in order to investigate the rebinding abilities and imprinting effect of CISs and NISs. Our previous results from Chapter-2 proved that post modification on MIPs were efficient in forming reversible covalent linkage with template molecules via thiol-disulfide exchange strategy. Hence, CISs and NISs underwent with previously reported modification step. However, silica particles (CISs and NISs) can be dissolved in alkali sodium hydroxide solutions. Therefore, CISs and NISs were treated with NaH in anhydrous acetonitrile, at the presence of four different template molecules benzyl thiosulfate salt, tertiary

benzyl thiosulfate salt, benzyl alcohol, and benzyl mercaptan. Rebinding and selectivity studies are not conducted in CIS3 and NIS3, because CIS3 releases very less amount of template and having too low amounts of available maximum binding sites.

The results from batch rebinding and selective studies on CIS1, NIS1, CIS2, and NIS2 are respectively illustrated in Figure 29, 30, and 31. The data is presented as a function of initial concentration (I) and amount of templates bound (B) to polymers.

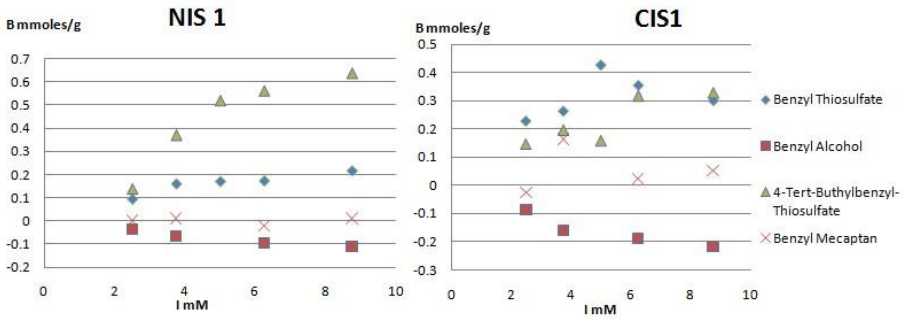

Figure 29: Results of batch rebinding studies of NIS1 and CIS1(Burri, 2010; Nielsen, 2010).

The results of rebinding abilities and selectivity of CIS1 and NIS1 are shown in Figure 29. The imprinted CIS1 silica polymers display the highest binding affinity towards benzyl thiosulfate salt since CIS1 may have the same spatial orientation towards benzyl mercaptan moieties, as well as being able to form reversible disulfide linkage via thiol-disulfide exchange with benzyl thiosulfate salt. NIS1 demonstrates highest binding affinities towards sodium tertiary butyl benzyl thiosulfate salt. This could be the expected since NIS1 were not prepared using template mediated (benzyl metcaptan) sol-gel process. The spatial effect of benzyl mercaptan cannot be obtained but the functional group is still the same. Hence NIS1 expresses higher binding towards sodium tertiary benzyl thiosulfate than it does on sodium benzyl thiosulfate salt.

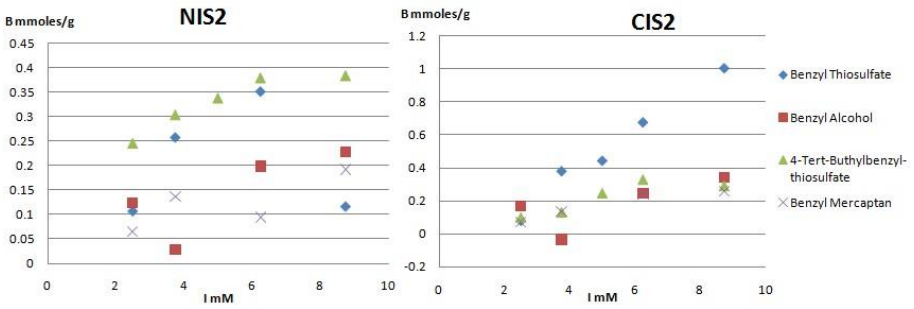

Figure 30: Results of batch rebinding studies of NIS2 and CIS2 (Burri, 2010; Nielsen, 2010).

The results of rebinding and selectivity of CIS2 and NIS2 are exhibited in the Figure 30. CIS2 reveals the highest binding affinities on sodium benzyl thiosulfate salt, and NIS2 bound mostly towards sodium tertiary butyl benzyl thiosulfate salt, which was expected from imprinting effect. CIS2 and NIS2 both showed low binding affinities to benzyl mercaptan. On the other hand, CIS1 evidenced the lowest affinity towards benzyl alcohol. CIS1 and NIS1 were expected to bind benzyl alcohol non-covalently. However, it has been not same in CIS2 and NIS2. These could be attributed into experimental deviations in recording values in CIS1 and NIS1 with benzyl alcohol.

In higher concentration range of incubated MIPs with sodium benzyl thiosulfate salt, it reached the saturation to maximum binding sites available in CIS1. However, binding amount of sodium benzyl thiosulfate in CIS2 exceeds Bmax, any such binding observed could be due to the unspecific template interactions with polymer networks (see Figure 31). Figure 31 represents the results of sodium benzyl thiosulfate salt binding with CIS1, CIS2, NIS1, and NIS2. The two imprinted CISs demonstrate expectedly higher binding affinities. However, highest binding affinity with CIS2 among other materials are found. The difference in binding sodium benzyl thiosulfate and sodium tertiary benzyl thiosulfate for CIS2 is higher than that for CIS1, as seen in Figure 29 and 30. This result was expected since CIS2 possesses a denser network than CIS1, due to this rigid network specific template binding moieties is more pronounced.

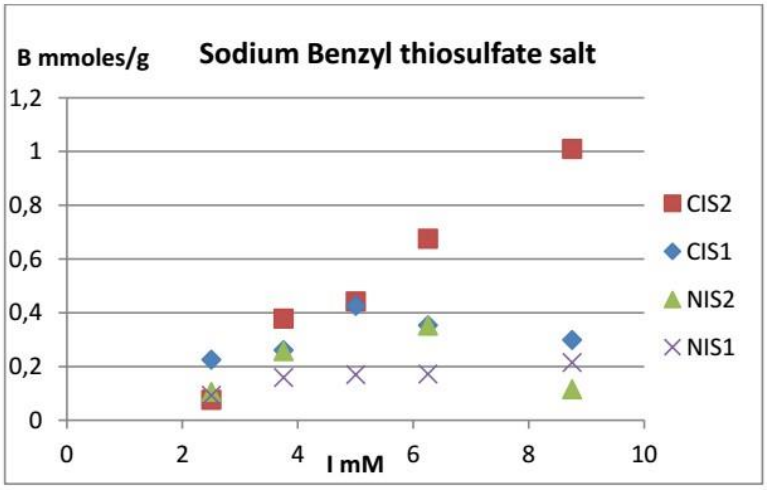

Figure 31: : Results of batch rebinding studies of sodium benzyl thiosulfate salt with CIS1, CIS2, NIS1, and NIS2 (Burri, 2010; Nielsen, 2010).

5.4 CONCLUSIONS AND PERSPECTIVES

Three different covalently imprinted silica network polymer particles were successfully synthesized by using the template mediated sol-gel process. A disulfide bond coupled functional monomer was successfully synthesized. With the covalently linked template, the functional monomer facilitates the sol-gel process at high temperatures. The optimal conditions and ratios between functional monomer and crosslinker were investigated with wide range of ratios employed in series of syntheses. With the minor change (sodium hydroxide replaced with sodium hydride), previously described novel post modification effect was successfully realized in the silica networks. The batch rebinding and selectivity studies demonstrate the imprinting effect and higher binding affinities with reversible forming covalent disulfide bonds. The template recognition carried out in protic solvents in silica networks can be used to develop novel sensor materials for artificial receptors and recognize them in biological systems. As per our knowledge, this is the first example using disulfide based covalent imprinting in silica networks.

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APPENDICES

Appendix A: Paper I: An Assay Study of Molecular Recognition of Amino Acids in Water: Covalent Imprinting of Cysteine.

Appendix B: Paper II: Covalent Imprinting and Covalent Rebinding of Benzyl Mercaptan: Towards a Facile Detection of Proteins.

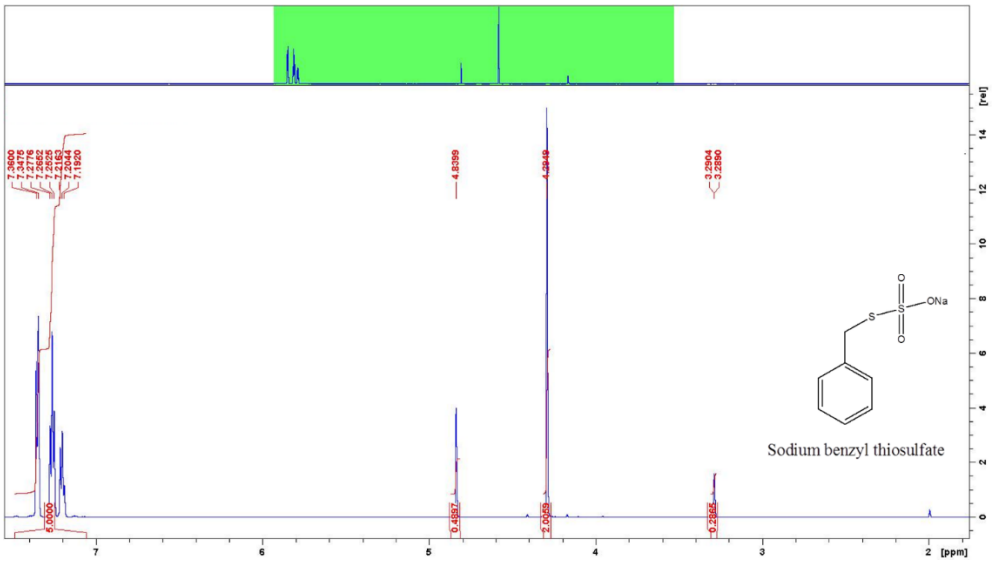
Appendix C: NMR Spectrums of Synthesized Compounds

Appendix D: HPLC Chromatograms and Standard Curves

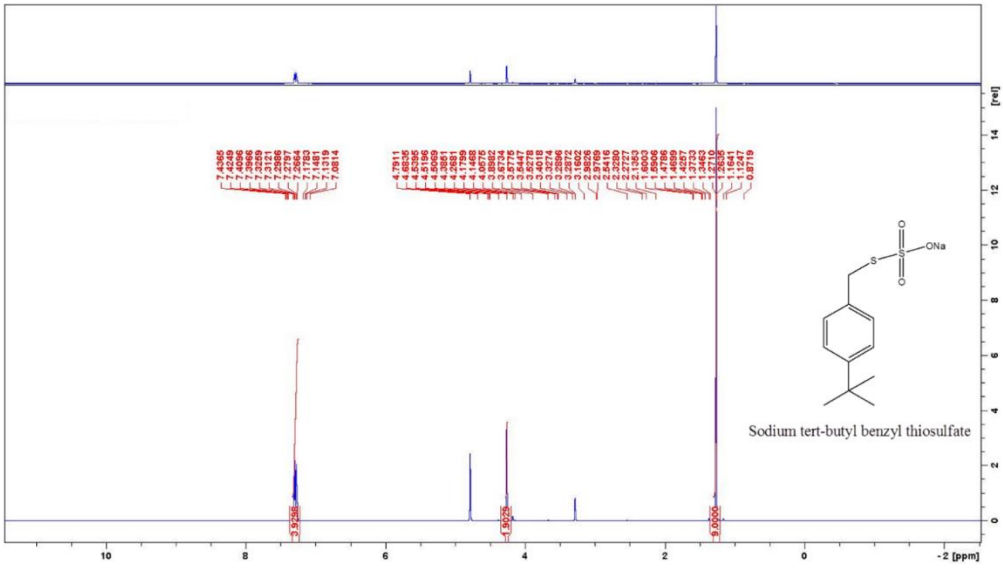
Appendix A: Paper I: An Assay Study of Molecular Recognition of Amino Acids in Water: Covalent Imprinting of Cysteine. ***Journal of Biomedical Science and Engineering*****. 2015, 8, 805- 814. DOI: 10.4236/jbise.2015.812077.**

Appendix B: Paper II: Covalent Imprinting and Covalent Rebinding of Benzyl Mercaptan: Towards a Facile Detection of Proteins. Analytical Letters, accepted and published on-line DOI: 10.1080/00032719.2016.1196694.

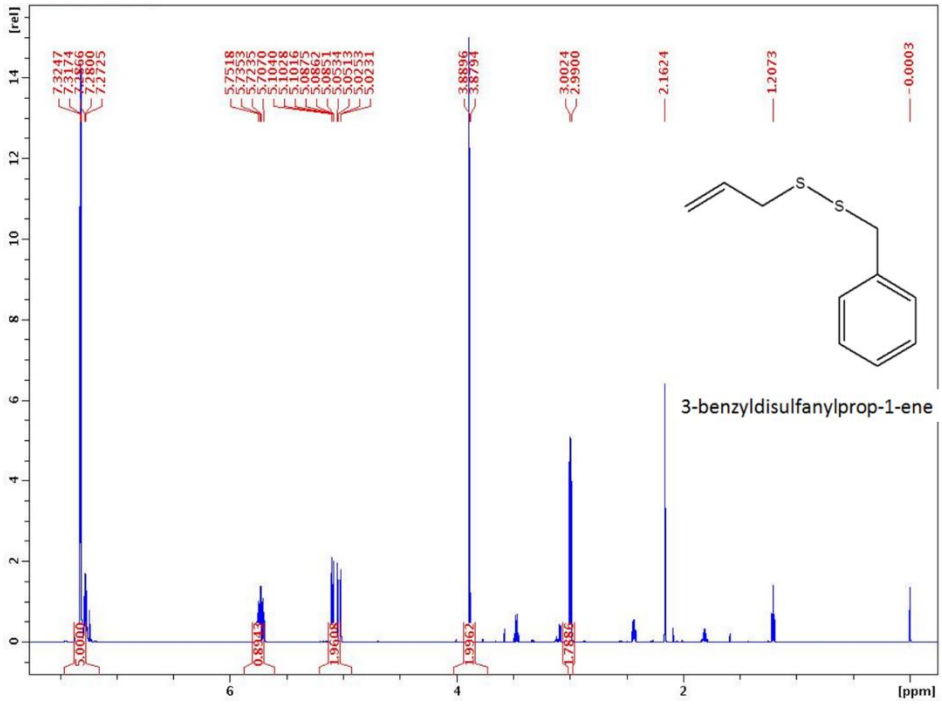
Appendix C: NMR Spectra's of synthesized compounds



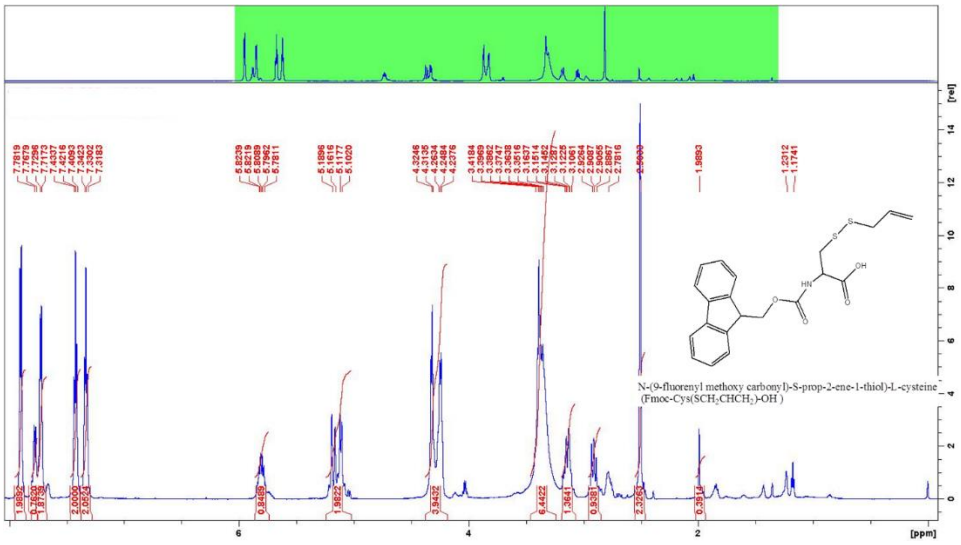
Appendix Figure 1: [1]H-NMR spectrum of Sodium benzyl thiosulfate salt (Compound 2a) in Chapter –2 and Chapter- 5



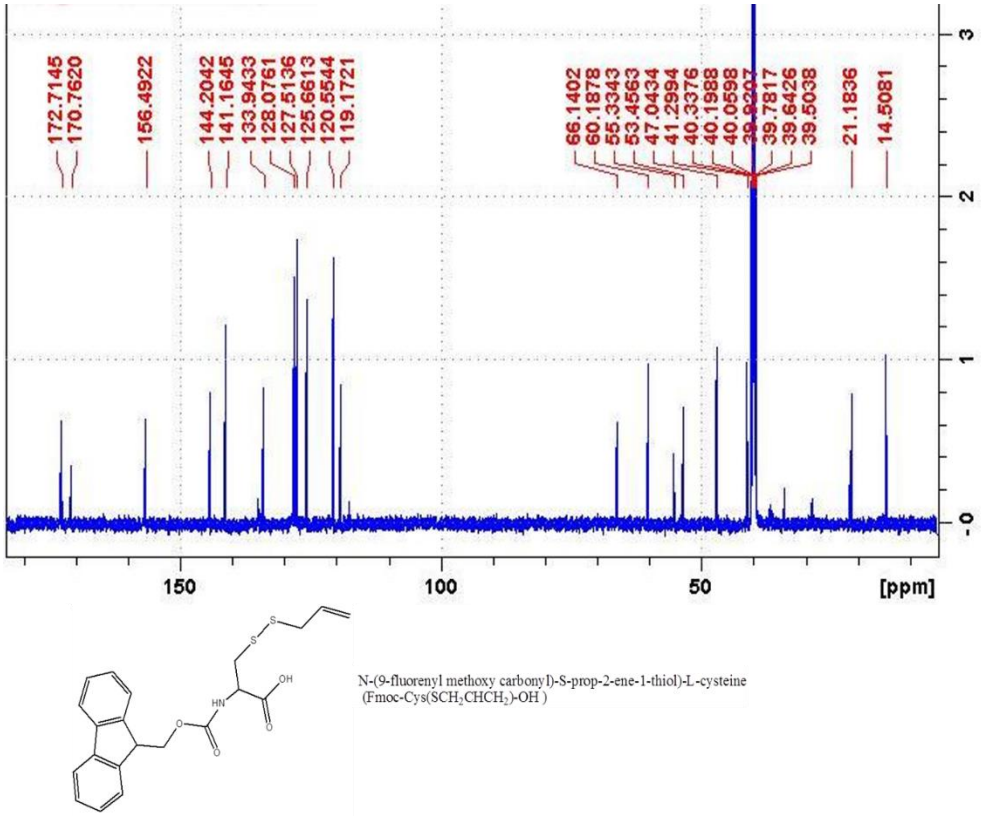
Appendix Figure 2: [1]H-NMR spectrum of Sodium 4-tert-butyl benzyl thiosulfate salt (Compound 2b) in Chapter –2 and Chapter- 5.



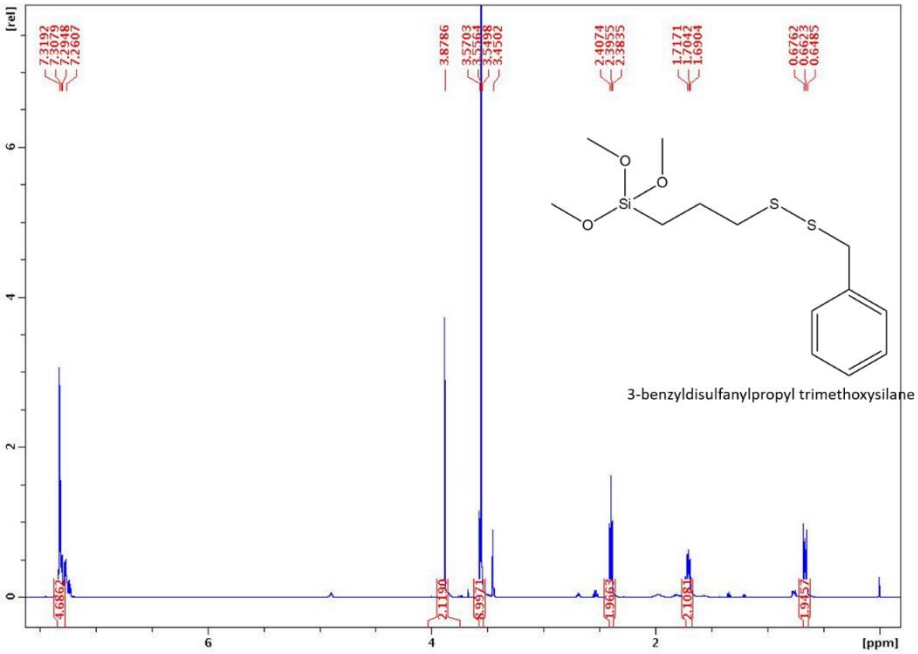
Appendix Figure 3: [1]H-NMR spectrum of functional monomer [3-benzyldisulfanylprop-1-ene (Compound 3)] in Chapter –2..



Appendix Figure 4: [1]H-NMR spectrum of functional monomer (9-fluorenyl methoxy carbonyl)-S-(1-propene-2-thiol)-L-Cysteine (Fmoc-Cys(SCH_2CHCH_2)-OH) (Compound 5)] in Chapter –3 and Chapter- 4.

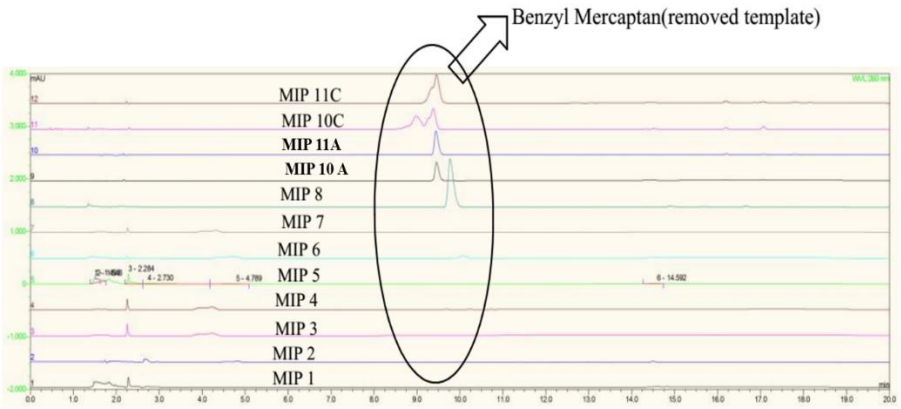


Appendix Figure 5: . ^{13}C- NMR spectra of functional monomer (9-fluorenyl methoxy carbonyl)-S-(1-propene-2-thiol)-L-Cysteine (Fmoc-Cys(SCH_2CHCH_2)-OH)] in Chapter –3 and Chapter- 5.

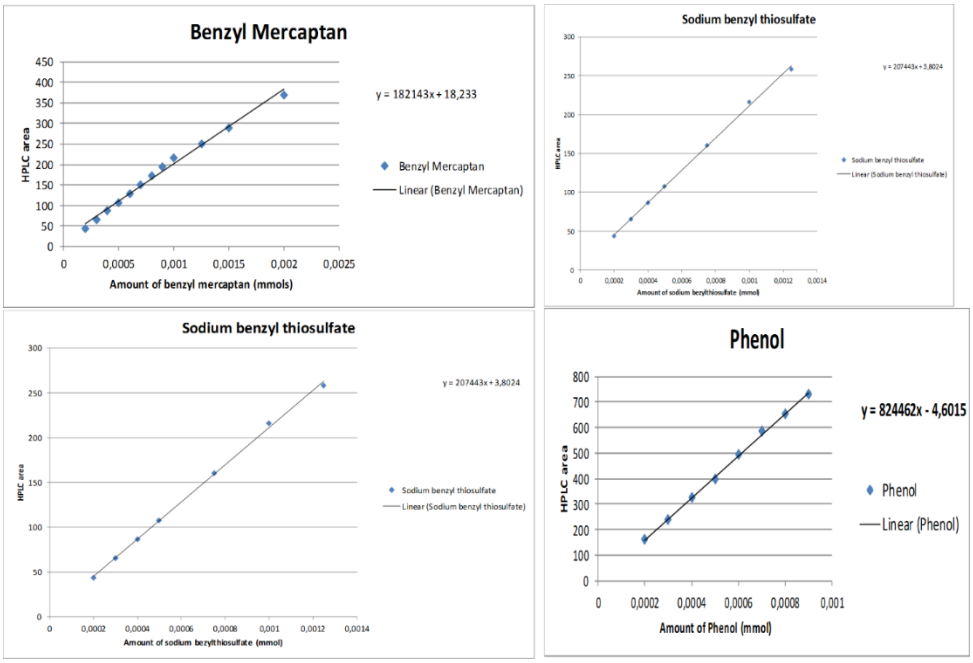


Appendix Figure 6: [1]H-NMR spectrum of functional monomer 3-benzyldisulfanylpropyl trimethoxysilane) in Chapter –5.

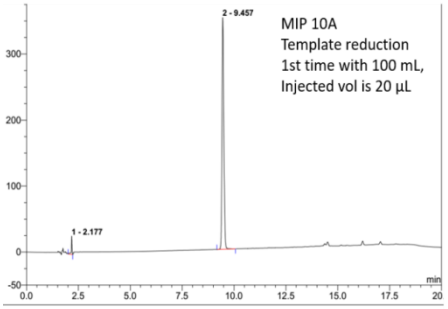
Appendix D: HPLC Chromatograms and Standard Curves



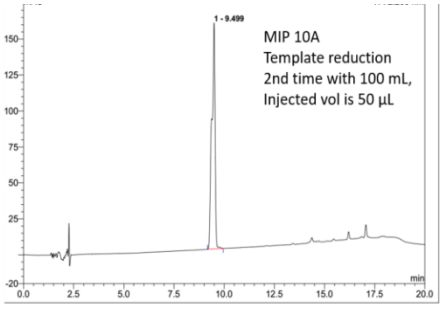
Appendix Figure 7: HPLC chromatograms of reduced templates from the MIP1- MIP11C. (From Chapter-2).



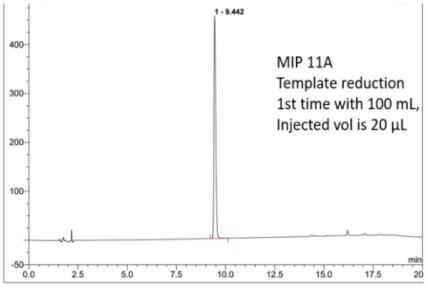
Appendix Figure 8: Standard curves of four different templates (Benzyl mercaptan, Sodium benzyl thiosulfate, Sodium 4-tert-butyl benzyl thiosulfate and Phenol) which were used for rebinding and selectivity studies in Chapter – 2.



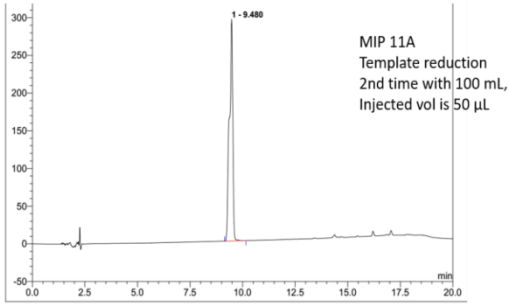
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.18	n.a.	27.722	1.028	2.74	n.a.	BMB
2	9.46	n.a.	350.863	36.457	97.26	n.a.	BMB
Total:			378.585	37.486	100.00	0.000	



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	9.50	n.a.	157.075	29.554	100.00	n.a.	BMB
otal:			157.075	29.554	100.00	0.000	



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	9.44	n.a.	454.675	48.856	100.00	n.a.	BMB
Total:			454.675	48.856	100.00	0.000	

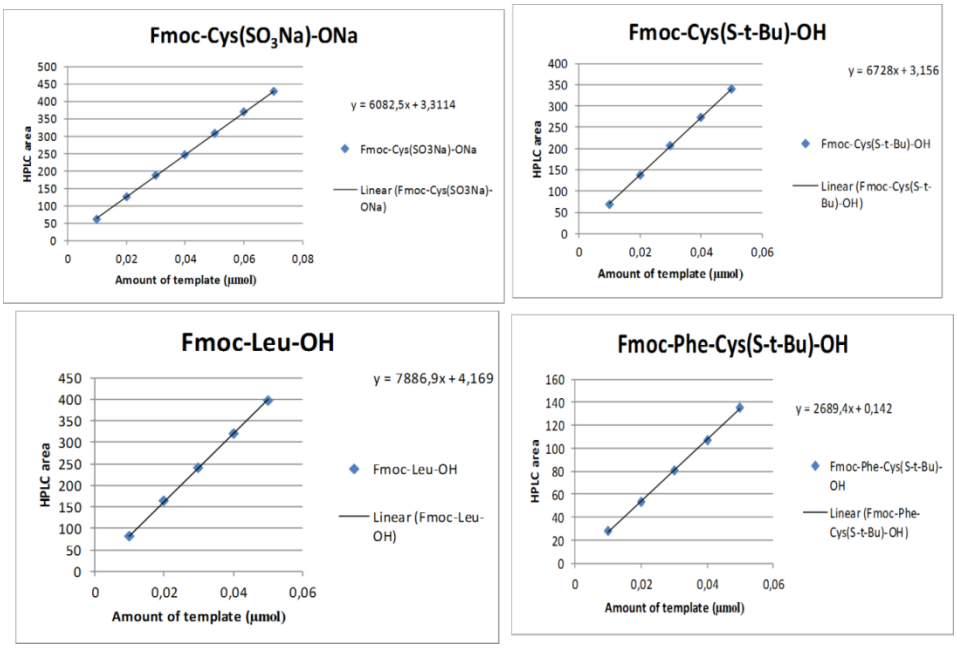


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	9.48	n.a.	294.089	54.772	100.00	n.a.	BMB
Total:			294.089	54.772	100.00	0.000	

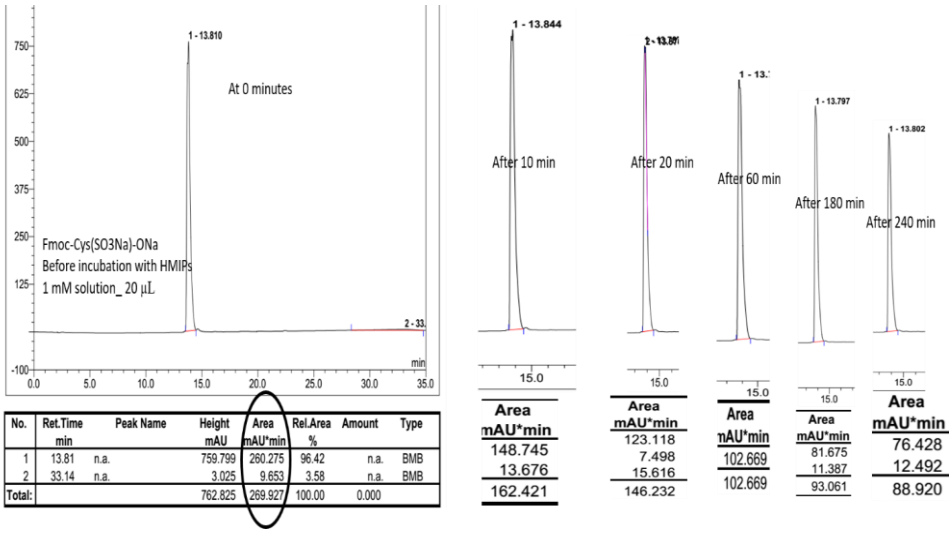
Appendix Figure 9: B_{max} quantification of MIP 10A and MIP11A. HPLC chromatograms of MIPs supernatant of reduced template. Left side- 1st time reduced supernatant and right side- second time repeated with the same procedure (From Chapter- 2).

Appendix Table 1: : Bmax of MIPs in calculated from the standard curve of corresponding compound. Here is the Bmax calculation of MIP 10A and MIP 11A. Standard curve

	Injected volume(µL) in to HPLC system	Amount (mmol) of template in 20 µL in supernatant	Amount (mmol) of template in 100mL in supernatant	Particles weight	Bmax (µmol/g)
MIP 10A- 1st time template removal	20	= (36-18,233)/182143 i.e. (0.0001 mmol in 20 µL)	= 0.500	3.94	157
MIP 10A- 2nd time template removal	50	= (29.5-18,233)/182143 i.e. (0.00006 mmol in 50 µL)	= 0.124		
MIP 10A- 1st time template removal	20	= (48.8-18,233)/182143 i.e. (0.00016 mmol in 20 µL)	= 0.84	6.14	202
MIP 11A- 2nd time template removal	50	= (54.7-18,233)/182143 i.e. (0.0002 mmol in 50 µL)	= 0.40		



Appendix Figure 10: Standard curves of four different templates (Fmoc-Cys(SO3Na)-ONa, Fmoc-Cys(S-t-bu)-OH, Fmoc-Leu-OH and Fmoc-Phe-Cys(S-t-bu)-OH) which were used for rebinding and selectivity studies in Chapter – 3.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	13.81	n.a.	759.799	260.275	96.42	n.a.	BMB
2	33.14	n.a.	3.025	9.653	3.58	n.a.	BMB
Total:			762.825	269.927	100.00	0.000	

Area mAU*min
148.745
13.676
162.421

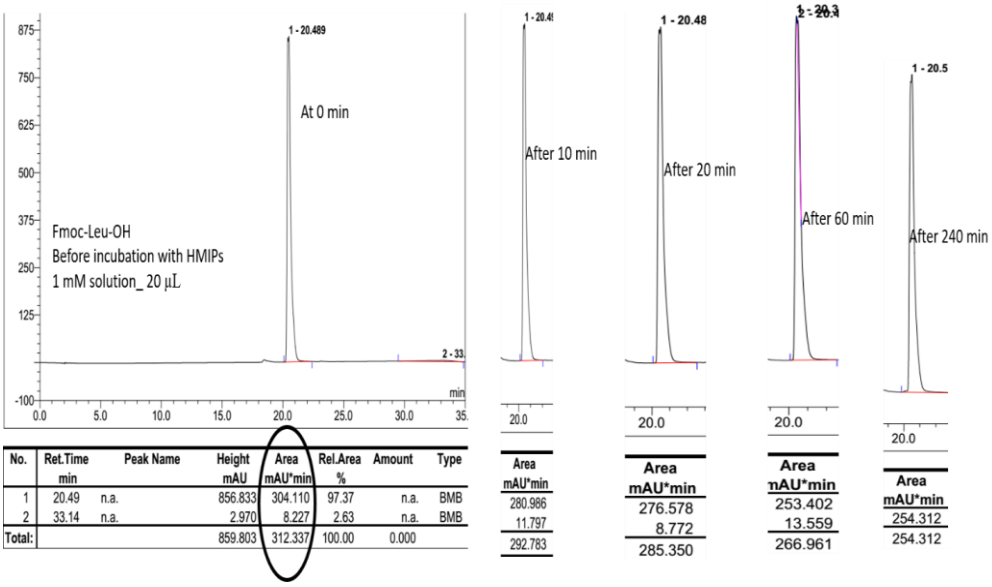
Area mAU*min
123.118
7.498
15.616
146.232

Area mAU*min
102.669
102.669

Area mAU*min
81.675
11.387
93.061

Area mAU*min
76.428
12.492
88.920

Appendix Figure 11: HPLC chromatograms of Fmoc-Cys(SO_3Na)-ONa incubated HMIP2 (covalent recognition) at different time points.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	20.49	n.a.	856.833	304.110	97.37	n.a.	BMB
2	33.14	n.a.	2.970	8.227	2.63	n.a.	BMB
Total:			859.803	312.337	100.00	0.000	

Area mAU*min
280.986
11.797
292.783

Area mAU*min
276.578
8.772
285.350

Area mAU*min
253.402
13.559
266.961

Area mAU*min
254.312
254.312

Appendix Figure 12: HPLC chromatograms of Fmoc-Leu-OH incubated HMIP2 (non-covalent recognition) at different time points.

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